

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N	A2	(11) International Publication Number: WO 98/26270 (43) International Publication Date: 18 June 1998 (18.06.98)
(21) International Application Number: PCT/US97/22601 (22) International Filing Date: 9 December 1997 (09.12.97) (30) Priority Data: 08/762,656 9 December 1996 (09.12.96) US		(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(71) Applicant: BIOLOG, INC. [US/US]; 3938 Trust Way, Hayward, CA 94545 (US). (72) Inventors: BOCHNER, Barry, R.; 22 Coleport Landing, Alameda, CA 94502 (US). NALEWAY, John, J.; 3247 Lincoln Street, Eugene, OR 97405-1927 (US). (74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).		

(54) Title: GEL MATRIX WITH REDOX PURPLE**(57) Abstract**

The present invention is directed to methods and compositions for the characterization of various microorganisms. In particular, the present invention is suited for the characterization of commonly encountered microorganisms (*e.g.*, *E. coli*, *S. aureus*, etc.), as well as commercially and industrially important organisms from various and diverse environments. For example, the present invention is particularly suited for the growth and characterization of the actinomycetes and fungi.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

GEL MATRIX WITH REDOX PURPLE

FIELD OF THE INVENTION

The present invention relates to growing and testing microorganisms in a multitest format which utilizes a gel forming matrix for the rapid screening of clinical and environmental cultures. The present invention is suited for the characterization of commonly encountered microorganisms (*e.g.*, *E. coli*, *S. aureus*, etc.), as well as commercially and industrially important organisms from various and diverse environments (*e.g.*, the present invention is particularly suited for the growth and characterization of the actinomycetes and fungi).

BACKGROUND OF THE INVENTION

Ever since the golden age of microbiology in the era of Koch and Pasteur, methods for identification of microorganisms have been investigated. Koch's experimental proof that microorganisms cause disease in the early 1800's, provided the impetus to study methods to grow and characterize harmful, as well as beneficial microorganisms. Koch's early experiments to determine the etiology of infectious diseases, led to methods for cultivation of microorganisms on the surface of solid media (*e.g.*, potato slices, see Koch, "Methods for the Study of Pathogenic Organisms," in T.D. Brock, Milestones in Microbiology, American Society for Microbiology, 1961, pp. 101-108; originally published as: "Zur Untersuchug von pathogenen Organismen." Mittheilungen aus dem Kaiserlichen Gesundheitsamte 1:1-48 [1881]). These studies eventually led to the development of agar as a culture medium component useful for producing solid media for growing isolated colonies of bacteria. To this day, isolated colonies are required (*i.e.*, "pure cultures") to biochemically identify organisms.

The field of diagnostic and clinical microbiology has continued to evolve, and yet, there remains a general need for systems that provide rapid and reliable biochemical identifications of microorganisms. In particular, it has been very difficult to develop an identification system which is capable of identifying various diverse types of organisms, ranging from the common isolate *Escherichia coli* to the less commonly encountered actinomycetes and fungi.

Methods and identification systems to characterize microorganisms widely used in industry for production of food and drink (*e.g.*, beer, wine, cheese, yogurt, etc.), the production of antibiotics (*e.g.*, penicillin, streptomycin, etc.), bioremediation of oil spills,

biological control of insect pests (e.g., *Bacillus thuringiensis*), and the production of recombinant proteins, are still needed. In addition, very few identification methods and systems have been developed for environmental use and there remains a need for simple and generally useful identification methods of many organisms. In particular, methods for identification and growth of the actinomycetes are lacking.

I. The Actinomycetes

The actinomycetes (members of the order *Actinomycetales*) include a large variety of organisms that are grouped together on the basis of similarities in cell wall chemistry, microscopic morphology, and staining characteristics. Nonetheless, this is a very diverse group of organisms. For example, genera within this group range from the strict anaerobes to the strict aerobes. Some of these organisms are important medical pathogens, while many are saprophytic organisms which benefit the environment by degrading dead biological or organic matter. While many of these organisms grow optimally at temperatures common in the environment (e.g., 25-27°C), some organisms are quite capable of growing at the body temperature of most mammals (e.g., approximately 35-37°C). However, two genera of medically important actinomycetes (*Thermomonospora* and *Micropolyspora*) are true thermophiles, capable of growing at temperatures ranging to 50°C.

Colonies may be bacterium-like (i.e., ranging from butyrous to waxy and glabrous), or fungus-like (i.e., heaped, leathery, membranous colonies that are covered with aerial hyphae). Many actinomycetes exhibit filamentous growth with mycelial colonies, and some actinomycetes cause chronic subcutaneous granulomatous abscesses much like those caused by fungi. Because of these similarities, the actinomycetes were long-regarded as fungi, rather than bacteria (see e.g., G.S. Kobayashi, "Actinomycetes: The fungus-like bacteria." in B.D. Davis *et al.*, Microbiology, 4th ed., J.B. Lippincott Co., New York, 1990), pp. 665-671).

Despite their similarities with the fungi, the actinomycetes have typical prokaryotic characteristics in terms of nucleoid and cell wall structure, antimicrobial sensitivity, the absence of sterols, motility by means of simple flagella, and long filaments of the diameter of bacteria (approximately 1 µm, compared to the larger fungal hyphae). Microscopically, the morphology of the aerobic actinomycetes varies widely between genera and species, although they are generally observed as gram-positive rods or branching filaments. Some genera never progress beyond a typical bacterium-like coccoid or bacillary form (e.g., *Rhodococcus sp.*), while others form filaments with extensive branching (e.g., *Nocardia*, *Streptomyces*).

Actinomadura, and *Nocardiosis*). Most are non-motile in their vegetative phase of growth. However, some genera tend to form branched filaments which eventually fragment into motile, flagellated cells (e.g., *Oerskovia* sp.) (see e.g., G. Land *et al.*, "Aerobic pathogenic *Actinomycetales*," in A. Balows *et al.*, Manual of Clinical Microbiology, 1991, pp. 340-359).

5 Most of the actinomycetes form spores, with the type of spore formation serving as a phylogenetic and taxonomic tool for separating the organisms into groups. The actinomycetes are highly diverse, with at least ten subgroups. They are also closely related to such organisms as the coryneform group (e.g., *Corynebacterium* sp.), the propionic acid bacteria (e.g., *Propionibacterium* sp.), and various obligate anaerobes (e.g., *Bifidobacterium*,

10 *Acetobacterium*, *Butyrivibrio*, and *Thermoanaerobacter*). The following table lists the organisms included in the suprageneric groups of actinomycetes as set forth in the most recent edition of Bergey's Manual, vol. 4. (Stanley T. Williams, editor of vol. 4; John G. Holt, editor in chief, Bergey's Manual® of Systematic Bacteriology, Williams & Wilkins, pp. 2334-2338 [1989]).

TABLE 1
Actinomycetes Groups

Number	Group	Representative Groups/Genera
I	Actinobacteria	Group A: <i>Agromyces</i> , <i>Aureobacterium</i> Group B: <i>Arthrobacter</i> , <i>Rothia</i> Group C: <i>Cellulomonas</i> , <i>Oerskovia</i> Group D: <i>Actinomyces</i> , <i>Arcanobacterium</i> Group E: <i>Arachnia</i> , <i>Pimelobacter</i> Group F: <i>Brevibacterium</i> Group G: <i>Dermatophilus</i>
II	Actinoplanetes	<i>Actinoplanes</i> , <i>Ampullariella</i> , <i>Micromonospora</i>
III	Maduromycetes	<i>Actinomadura pusilla</i> group, <i>Microbispora</i> , <i>Streptosporangium</i>
IV	Micropolysporas	<i>Actinopolyspora</i> , <i>Faenia</i> , <i>Saccharomonospora</i>
V	Multilocular Sporangia	<i>Frankia</i> , <i>Geodermatophilus</i>
VI	Nocardioforms	<i>Nocardia</i> , <i>Rhodococcus</i> , <i>Caseobacter</i>
VII	Nocardioides	<i>Nocardioides</i>
VIII	Streptomycetes	<i>Streptomyces</i> , <i>Streptoverticillium</i> , <i>Kineosporia</i>
IX	Thermomonosporas	<i>Thermomonospora</i> , <i>Nocardiopsis</i> , <i>Actinomadura madurae</i> group
X	Other Genera	<i>Glycomyces</i> , <i>Kitasatosporia</i> , <i>Spirillospora</i> , <i>Thermouctinomyces</i>

Although these organisms may often be identified to the genus level based on their morphology at the time of primary isolation, organisms that have been repeatedly transferred in the laboratory often do not retain their typical morphologic characteristics and must be identified biochemically, or by analysis of their membrane fatty acid composition. Serological methods for identification and differentiation are rarely used, due to the extensive degree of cross-reactivity among the actinomycetes (*see e.g.*., G.S. Kobayashi, *supra*, at p. 666).

II. Importance of the Actinomycetes as Pathogens

Many of these organisms are soil-dwellers, with relatively little pathogenic capabilities. Indeed, the actinomycetes are among the most abundant of organisms in the soil, where they serve the important function of breaking down proteins, cellulose, and other organic matter.

5 Nonetheless, some *Actinomyces*, *Nocardia*, and *Streptomyces* species are associated with diseases of medical and veterinary importance, especially in immunocompromised individuals. The spectrum of disease caused by the actinomycetes is extremely broad, with pathology that is dependent upon a combination of organism type, tissue involved, and the immune status of

10 the host. In immunocompetent humans, the most common diseases are a non-invasive, acute or chronic allergic respiratory syndrome (e.g., farmer's lung), and mycetoma. In immunocompromised individuals, infection often begins in the lung as an acute to chronic suppurative process, which may progress to cavitation and multi-lobular pulmonary disease. In these patients, infection may spread to other organ systems. Importantly, these organisms have a predilection for the central nervous system.

15 Several species of *Actinomyces* have been associated with actinomycosis in humans and other animals, with *A. israelii* being the most common human isolate, and *A. bovis* the most common cattle isolate. Actinomycosis is usually characterized by chronic, destructive abscesses of connective tissues. Abscesses expand into the neighboring tissues and eventually produce burrowing, tortuous sinus tracts to the surface of the skin, where purulent material is
20 discharged. In cattle, the lesions are characteristically large abscesses of the lower jaw (hence the common name of the disease, "lumpy jaw"), usually with extensive bone destruction. As with most saprophytic organisms that occasionally cause disease, actinomycosis is not transmissible from person to person, nor between humans and other animals. Indeed, it is difficult to establish infection in laboratory animals.

25 For *in vitro* growth in the laboratory, these pathogenic organisms tend to be microaerophilic (e.g., require a decreased oxygen tension for optimum growth), require rich growth media, optimum incubation temperatures of 37°C, and about 7 days of incubation. Although actinomycetes are soil organisms, actinomycosis is usually caused by endogenous organisms that have colonized the individual, rather than organisms from the environment.
30 The organism is usually a commensal, which can be cultured from the tonsils of most humans, and is almost always present in teeth and gum scrapings. The conditions that lead to invasiveness are not well characterized, but may be multi-factorial, as actinomycotic infections

are often mixed. with various organisms (e.g., *Haemophilus actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium*, and *Bacteroides*) also present.

In contrast to the *Actinomyces*, diseases due to *Nocardia sp.* are associated with infection of the individual with soil organisms, rather than endogenous commensals.

5 *Nocardia* are among the most clinically important actinomycetes, as they are responsible for the majority of disease associated with this group of organisms. Indeed, the term "nocardiosis" is often used synonymously for pulmonary and disseminated infection caused by any of the aerobic actinomycetes (see e.g., G. Land *et al.*, "Aerobic Pathogenic *Actinomycetales*," in A. Balows *et al.*, Manual of Clinical Microbiology, 5th ed., American
10 Society for Microbiology, Washington, D.C., 1991, pages 340-359).

There are two common forms of disease associated with *Nocardia sp.*, namely, pulmonary nocardiosis resulting from inhalation of the organism, and mycetoma, which is characterized by chronic subcutaneous abscesses resulting from contamination of skin wounds. These infections are usually serious, especially as they are frequently seen in association with
15 immunosuppression or chronic underlying diseases (e.g., carcinoma, chronic granulomatous disease, Hodgkin's disease, and leukemia). Once clinically evident, the progression of nocardiosis tends to be progressive and fatal, with approximately 50% of patients dying, even with aggressive therapy (see e.g., G.S. Kobayashi, "Actinomycetes: The Fungus-Like Bacteria, in B.D. Davis *et al.* (eds.), Microbiology, 4th ed., J.B. Lippincott Co., Philadelphia
20 [1990], pages 665-671).

The *Nocardia* are aerobic organisms which grow on relatively simple media over a wide temperature range. As with the mycobacteria, growth in liquid media usually results in the production of a dry, waxy pellicle on the surface of the media. The two species most commonly associated with human disease, *N. brasiliensis* and *N. asteroides*, share many other
25 characteristics with the mycobacteria. For example, they are somewhat acid-fast, more easily stained with fuchsin, and their cell walls contain components characteristic of mycobacteria and corynebacteria (e.g., mycolic acid residues). Unlike the great majority of organisms, the somewhat harsh methods used to isolate mycobacteria (e.g., treatment of samples with N-acetyl-L-cysteine, and sodium hydroxide) are often successful for isolation of *Nocardia*.
30 Extensive serologic cross-reactions in agglutination and complement fixation tests further indicate the relatedness of these groups of organisms.

The *Streptomyces* are also sometimes associated with actinomycotic abscesses. Mycetomas caused by streptomycetes are clinically indistinguishable from those caused by

other actinomycetes. However, identification of these organisms can be critical, as they are generally not susceptible to antimicrobial agents. Therefore, treatment often entails surgical removal of the affected area or amputation.

Other members of the actinomycetes are capable of causing disease, including allergic respiratory disease ("farmer's lung"), which occurs in agricultural workers who inhale dust from moldy plant material. This syndrome has been associated with at least three thermophilic actinomycetes (*Thermopolyspora polyspora*, *Micromonospora vulgaris*, and *Micropolyspora faeni*). This disease is very similar to that caused by inhalation of allergens produced by various fungi, particularly *Aspergillus* sp.

In addition to the pathogenic potential of this group of organisms, there is also great interest in the particular genera which produce antimicrobial compounds.

III. Industrial Importance of the Actinomycetes

Ever since Waksman isolated actinomycin in 1940, and streptomycin in 1943, the streptomycetes have attracted a large amount of attention (see e.g., G.S. Kobayashi, *et al.*, at p. 671). Thousands of soil samples collected world-wide have resulted in the identification of over 90% of the therapeutically useful antibiotics (see e.g., G.S. Kobayashi, "Actinomycetes: The Fungus-Like Bacteria, in B.D. Davis *et al.* (eds.), *Microbiology*, 4th ed., J.B. Lippincott Co., Philadelphia [1990], pages 665-671). The interest in improving antibiotic qualities and yields has resulted in various studies on this group of organisms, including improved methods for their growth and characterization.

It is important that strains be differentiated in screening programs to identify antibiotic activities, so that redundant testing is avoided. In addition, differentiation facilitates determination of taxonomic relationships which may lead to other organisms with promising activities. Unfortunately, testing of these organisms is often very difficult. Because they grow as filaments, they have a strong tendency to form clumps of mycelia which makes them much more difficult to handle, both in liquid cultures and on solid or semi-solid agar media. Furthermore, because of their complex life cycle which involves sporulation and germination, it is very difficult to obtain cultures which perform consistently in metabolic and biochemical testing programs. In addition, the presence of spores and the potential for their inhalation, represents a safety hazard to personnel responsible for the cultivation and characterization of these organisms, especially in settings where large-scale growth is necessary (e.g., antimicrobial production).

These growth characteristics also contribute to the difficulties associated with determining the susceptibility of the actinomycetes to antimicrobial compounds. The most frequently used testing methods are a modified Kirby-Bauer disk diffusion method agar dilution, and a minimal inhibitory concentration (MIC) method (*see e.g.*, G. Land *et al.*, "Aerobic Pathogenic *Actinomycetales*," in A. Balows *et al.*, Manual of Clinical Microbiology, 5th ed., American Society for Microbiology, Washington, D.C., 1991, at p. 356). However, the success of these methods is contingent upon the production of a homogenized suspension for use as a standardized inoculum. Most commonly, agitation with sterile glass beads or a tissue homogenizer is used to prepare a homogenous suspension that can then be diluted to a 0.5 McFarland standard prior to inoculating the test media (*see e.g.*, G. Land *et al.*, "Aerobic Pathogenic *Actinomycetales*," in A. Balows *et al.*, Manual of Clinical Microbiology, 5th ed., American Society for Microbiology, Washington, D.C., 1991, pages 340-359). These methods involving physical homogenization are very labor-intensive and tedious, and they result in damage, fragmentation, and death of some fraction of the cells. Furthermore, the additional manipulation required to produce a homogenous suspension prior to inoculation increases the risk of contamination of laboratory personnel and the laboratory environment.

Therefore, what is needed is a safe, reliable, easy-to-use system for the characterization and testing of these medically and industrially important organisms. In particular what is need is a rapid method that is readily automatable and useful in various settings (*e.g.*, clinical, veterinary and environmental laboratories, and industry).

SUMMARY OF THE INVENTION

The present invention relates to growing and testing microorganisms in a multitest format which utilizes a gel forming matrix for the rapid screening of clinical and environmental cultures. In particular, the present invention is suited for the characterization of commonly encountered microorganisms (*e.g.*, *E. coli*, *S. aureus*, etc.), as well as commercially and industrially important organisms from various and diverse environments. For example, the present invention is particularly suited for the growth and characterization of bacteria, as well as the actinomycetes and fungi (*e.g.*, yeasts and molds).

In one embodiment, the present invention provides methods for testing microorganisms comprising the steps of: providing a testing means comprising redox purple and one or more test substrates; introducing microorganisms into the testing means; and detecting the response

of the microorganism to the one or more test substrates. In a preferred embodiment, the testing substrates are selected from the group consisting of carbon sources and antimicrobials.

In an alternate embodiment, the testing means further comprises one or more gel-initiating agents. In a preferred embodiment, the gel-initiating agent comprises cationic salts.

5 In another alternative embodiment, the testing means further comprises one or more gelling agents. In a preferred embodiment, the microorganisms are in an aqueous suspension. In another preferred embodiment, the aqueous suspension further comprises one or more gelling agents. ~~It is contemplated that various gelling agents will be used with the present invention,~~ including, but not limited to agar, Gelrite™, carrageenan, and alginic acid.

10 In one embodiment of the method, the microorganisms are bacteria, while in another embodiment, the microorganisms are fungi. It is also contemplated that the methods of the present invention will be used with members of the Order *Actinomycetales*.

It is contemplated that various testing means will be used in the present invention. In one preferred embodiment, the testing means comprises at least one microplate, in an
15 alternative embodiment, the testing means comprises at least one microcard. In yet another embodiment, the testing means comprises at least one petri plate.

The present invention also provides a kit, comprising redox purple and one or more test substrates. In a preferred embodiment, the test substrates are selected from the group consisting of carbon sources and antimicrobials. In another preferred embodiment, the kit
20 further comprises one or more gel-initiating agents. In a particularly preferred embodiment, the gel initiating agent comprises cationic salts. In an alternative preferred embodiment, the kit further comprises one or more gelling agents. In another preferred embodiment, the gelling agent is selected from the group consisting of agar, Gelrite™, carrageenan, and alginic acid.

25 In another embodiment, the kit further comprises a suspension of microorganisms. In one preferred embodiment, the kit further comprises a testing means. It is contemplated that various testing means formats will be used successfully in various embodiments of the kits of the present invention, including microplates, microcards, petri plates, and any other suitable support in which the testing reaction can occur.

30 In yet another embodiment, the present invention provides a kit, comprising redox purple and one or more gelling agents. It is contemplated that various gelling agents will be used successfully in the various embodiments of the kits of the present invention, including but not limited to agar, Gelrite™, carrageenan, and alginic acid. In one preferred

embodiment, the kit further comprises one or more gel-initiating agents. In a particularly preferred embodiment, the gel-initiating agent comprises cationic salts. In an alternative embodiment, the kit further comprises a suspension of microorganisms.

In an alternative embodiment, the kit further comprises one or more test substrates. It is contemplated that the test substrates included in the kit of the present invention be selected from the group consisting of carbon sources and antimicrobials. In yet another embodiment, the kit further comprises a testing means. It is contemplated that various testing means formats will be used successfully in various embodiments of the kits of the present invention, including microplates, microcards, petri plates, and any other suitable support in which the testing reaction can occur.

The present invention describes test media and methods for the growth, isolation, and presumptive identification of microbial organisms. The present invention contemplates compounds and formulations, as well as methods particularly suited for the detection and presumptive identification of various diverse organisms.

In order to characterize or identify organisms present in a sample, the present invention combines a gel-forming suspension with microorganisms that are already in the form of a pure culture. This is in contrast to the traditional pour plate method which involves heated agar and a sample that contains a mixed culture (*see e.g.*, J.G. Black, Microbiology: Principles and Applications, 2d ed., Prentice Hall, Englewood Cliffs, NJ, p. 153 [1993]; and American Public Health Association, Standard Methods for the Examination of Water and Wastewater, 16th ed., APHA, Washington, D.C., pp. 864-866 [1985]).

It is also in contrast to the pour plate method of Roth (U.S. Patent Nos. 4,241,186, and 4,282,317), which utilizes a solidifying pectin substance. In the present invention, colloidal gel-forming substances are used at low concentrations, forming soft gels or viscous colloidal suspensions that do not need to, and in fact work best, when not completely solidified into a rigid gel.

In one embodiment, the present invention provides a method for introducing microorganisms into a testing device, comprising the steps of providing a testing device comprising a plurality of testing wells or compartments, wherein each compartment contains one or more gel-initiating agents; preparing a suspension comprising a pure culture of microorganisms and an aqueous solution containing a gelling agent, under conditions such that the suspension remains ungelled; and introducing the suspension into the testing device under

conditions such that the suspension contacts the gel-initiating agents present in the compartments and results in the production of a gel or colloidal matrix.

In another embodiment, the present invention provides a method for testing microorganisms comprising the steps of providing a testing device comprising a plurality of testing compartments, wherein the compartments contain a testing substrate and one or more gel-initiating agents; preparing a suspension comprising a pure culture of microorganisms and an aqueous solution comprising a gelling agent under conditions such that the suspension remains ungelled; ~~introducing the suspension into the compartments of the testing device~~

~~under conditions such that the suspension forms a gel matrix within the compartment; and detecting the response of the microorganisms to the testing substrate.~~ In one preferred embodiment, the testing device is a microplate.

It is contemplated that the microorganisms tested in this method will be bacteria, including members of the Order *Actinomycetales*, or fungi (e.g., yeasts and molds).

In one embodiment, the gelling agent is selected from the group consisting of Gelrite™, carrageenan, and alginic acid. In a particularly preferred embodiment, the gelling agent is carrageenan which contains predominantly iota-carrageenan. In one embodiment, the gel-initiating agent comprises cationic salts.

In one embodiment, the testing substrates are selected from the group consisting of carbon sources and antimicrobials. In yet another embodiment, the method further includes a colorimetric indicator, wherein the colorimetric indicator is selected from the group consisting of chromogenic substrates, oxidation-reduction indicators, and pH indicators.

In yet another embodiment, the present invention encompasses a kit for growth and identification of microorganisms comprising: a testing device comprising a plurality of testing compartments containing one or more gel-initiating agents; and an aqueous solution comprising a gelling agent. In one preferred embodiment, the testing compartments further contain testing substrates, such as carbon sources and antimicrobials. In one embodiment, the gel-initiating agent comprises cationic salts.

In one embodiment of this kit, the testing device is a microplate. In a preferred embodiment, the kit contains a gelling agent that is selected from the group consisting of Gelrite™, carrageenan, and alginic acid. In one preferred embodiment, the gelling agent is a carrageenan which predominantly contains the iota form of carrageenan. In one embodiment, the gel-initiating agent comprises cationic salts.

It is contemplated that the kit of the present invention will be used with microorganisms such as bacteria, including members of the Order *Actinomycetales*, as well as fungi (e.g., yeasts and molds).

It is also contemplated that the kit will also include a colorimetric indicator selected from the group consisting of chromogenic substrates, oxidation-reduction indicators, and pH indicators.

In an alternative embodiment, the present invention comprises a kit for characterizing and identifying microorganisms comprising: a microplate testing device containing a plurality of compartments, wherein the compartments contain one or more gel-initiating agents and one or more testing substrates, wherein the testing substrates are selected from the group consisting of antimicrobials and carbon sources and an aqueous suspension comprising a gelling agent.

In one embodiment of this kit, the testing device is a microplate. In a preferred embodiment, the kit contains a gelling agent that is selected from the group consisting of Gelrite™, carrageenan, and alginic acid. In one preferred embodiment, the gelling agent is a carrageenan which predominantly contains the iota form of carrageenan. In one embodiment, the gel-initiating agent comprises cationic salts.

It is contemplated that the kit of the present invention will be used with microorganisms such as bacteria, including members of the Order *Actinomycetales*, as well as fungi (e.g., yeasts and molds).

It is also contemplated that the kit will include a colorimetric indicator selected from the group consisting of chromogenic substrates, oxidation-reduction indicators, and pH indicators.

DESCRIPTION OF THE FIGURES

Figure 1 is an exploded perspective view of one embodiment of the device of the present invention.

Figure 2 is a top plan view of the device shown in Figure 1.

Figure 3 is a cross-sectional view of the device shown in Figure 2 along the lines of 3-

-3.

Figure 4 is a bottom plan view of the device shown in Figure 1.

Figure 5 shows the synthesis pathway of redox purple.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated in part on the discovery that various organisms contained within a gel matrix may be differentiated based on differential biochemical reactions. The present invention incorporates a multiple test format in a testing device, for presumptive and rapid microbiological screening of various clinical, veterinary, industrial and environmental specimens. It is also intended that the present invention will be useful for definitive identification and diagnosis. In particular, this invention is suitable for the rapid biochemical testing of actinomycetes. ~~It is not intended that the invention be limited to a~~ particular genus, species nor group of organisms. This medium and method are particularly targeted toward some of the most economically important organisms, as well as species of clinical importance.

The present invention contemplates an indicator plate similar in structure to microtiter plates ("microplates" or "MicroPlates™") commonly used in the art and commercially available from numerous scientific supply sources (*e.g.*, Biolog, Fisher, etc.). It is contemplated that the present invention be used with various gelling agents, including but not limited to alginate, carrageenan, and gellan gum (*e.g.*, Gelrite™).

Because the organisms are trapped within the gel matrix, the present invention is a great improvement over standard microplate testing methods in which liquid cultures are used. Unlike the liquid format, the gel matrix of the present invention does not spill from the microplate, even if the plate is completely inverted. This safety consideration highlights the suitability of the present invention for use with organisms that are easily aerosolized. It is also contemplated that the present invention is highly useful in the educational setting, where safety is a primary concern. The present invention permits novices to work with bacteria and study their biochemical characteristics with a reduced chance of contamination, as compared to other testing systems.

As various organisms may be characterized using the present invention, it is not intended that the choice of primary isolation media be limited to particular formulae. However, in a preferred embodiment, organisms such as the actinomyces are grown on an agar medium which stimulates the production of aerial conidia. This greatly facilitates the harvesting of organisms for inoculation in the present invention.

In one embodiment, a microtiter (*e.g.*, microplate) format is used. In this embodiment, the gel-forming matrix containing suspended microorganisms is used to inoculate the wells of a microtiter plate or another receptacle. At the time of inoculation, the gel-forming matrix is

in liquid form, allowing for easy dispensing of the suspension into the compartments. These compartments contain dried biochemicals and cations. Upon contact of the gel-forming matrix with the cations, the suspension solidifies to form a soft gel, with the organisms evenly distributed throughout. This gel is sufficiently viscous or rigid that it will not fall out of the microtiter plate should the plate be inverted.

In another embodiment, a microcard format is used. As shown in Figures 1-4, one embodiment of the device of the present invention comprises a housing (100) with a liquid entry port through which the sample is introduced. The housing further contains a channel (110) providing communication to a testing region (120) so that a liquid (not shown) can flow into a plurality of wells or compartments (130). The channel (110) is enclosed by the surface of a hydrophobic, gas-venting membrane (140) adapted for forming one surface of the wells (130) and attached to one side of the housing (100). The housing (100) can be sealed on its other side by a solid base (150). In other embodiments, a flexible tape (not shown) may be substituted for the solid base (150) or the solid base (150) may be molded so as to be integral with the housing (100).

After filling the device with the gel-forming matrix containing microorganisms, (not shown) an optional non-venting material such as tape (160) can be adhered to the outer surface of the gas-venting membrane (140) to seal it against evaporation of the gel matrix within the device through the gas-venting membrane. At the time of delivery, the gel-forming matrix with suspended organisms is in liquid form. Once the liquid comes into contact with the compounds present in the testing region, a gel matrix is produced, trapping the suspended microorganisms.

Although embodiments have been described with some particularity, many modifications and variations of the preferred embodiment are possible without deviating from the invention.

Definitions

The terms "sample" and "specimen" in the present specification and claims are used in their broadest sense. On the one hand, they are meant to include a specimen or culture. On the other hand, they are meant to include both biological and environmental samples. These terms encompasses all types of samples obtained from humans and other animals, including but not limited to, body fluids such as urine, blood, fecal matter, cerebrospinal fluid (CSF).

semen, and saliva, as well as solid tissue. These terms also refers to swabs and other sampling devices which are commonly used to obtain samples for culture of microorganisms.

Biological samples may be animal, including human, fluid or tissue, food products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste.

5 Environmental samples include environmental material such as surface matter, soil, water, and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, disposable, and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

10 Whether biological or environmental, a sample suspected of containing microorganisms may (or may not) first be subjected to an enrichment means to create a "pure culture" of microorganisms. By "enrichment means" or "enrichment treatment," the present invention contemplates (i) conventional techniques for isolating a particular microorganism of interest away from other microorganisms by means of liquid, solid, semi-solid or any other culture medium and/or technique, and (ii) novel techniques for isolating particular microorganisms
15 away from other microorganisms. It is not intended that the present invention be limited only to one enrichment step or type of enrichment means. For example, it is within the scope of the present invention, following subjecting a sample to a conventional enrichment means, to subject the resultant preparation to further purification such that a pure culture of a strain of a species of interest is produced. This pure culture may then be analyzed by the medium and
20 method of the present invention.

As used herein, the term "culture" refers to any sample or specimen which is suspected of containing one or more microorganisms. "Pure cultures" are cultures in which the organisms present are only of one strain of a particular genus and species. This is in contrast to "mixed cultures," which are cultures in which more than one genus and/or species of
25 microorganism are present.

As used herein, the term "organism" is used to refer to any species or type of microorganism, including but not limited to bacteria, yeasts and other fungi. As used herein, the term fungi, is used in reference to eukaryotic organisms such as the molds and yeasts, including dimorphic fungi.

30 As used herein, the term "spore" refers to any form of reproductive elements produced asexually (e.g., conidia) or sexually by such organisms as bacteria, fungi, algae, protozoa, etc. It is also used in reference to structures within microorganisms such as members of the genus *Bacillus*, which provide advantages to the individual cells in terms of survival under harsh

environmental conditions. It is not intended that the term be limited to any particular type or location of spores, such as "endospores" or "exospores." Rather, the term is used in the very broadest sense.

As used herein, the terms "microbiological media" and "culture media," and "media" refer to any substrate for the growth and reproduction of microorganisms. "Media" may be used in reference to solid plated media which support the growth of microorganisms. Also included within this definition are semi-solid and liquid microbial growth systems including those that incorporate living host organisms, as well as any type of media.

As used herein, the term "carbon source" is used in reference to any compound which may be utilized as a source of carbon for bacterial growth and/or metabolism. Carbon sources may be in various forms, including, but not limited to polymers, carbohydrates, acids, alcohols, aldehydes, ketones, amino acids, and peptides.

As used herein, the term "nitrogen source" is used in reference to any compound which may be utilized as a source of nitrogen for bacterial growth and/or metabolism. As with carbon sources, nitrogen sources may be in various forms, such as free nitrogen, as well as compounds which contain nitrogen, including but not limited to amino acids, peptones, vitamins, and nitrogenous salts.

As used herein, the term "antimicrobial" is used in reference to any compound which inhibits the growth of, or kills microorganisms. It is intended that the term be used in its broadest sense, and includes, but is not limited to compounds such as antibiotics which are produced naturally or synthetically. It is also intended that the term includes compounds and elements that are useful for inhibiting the growth of, or killing microorganisms.

As used herein, the term "testing substrate" is used in reference to any carbon and/or nitrogen source that may be utilized to differentiate bacteria based on biochemical characteristics. For example, one bacterial species may utilize one testing substrate that is not utilized by another species. This utilization may then be used to differentiate between these two species. It is contemplated that numerous testing substrates be utilized in combination. Testing substrates may be tested individually (*e.g.*, one substrate per testing well or compartment, or testing area) or in combination (*e.g.*, multiple testing substrates mixed together and provided as a "cocktail").

Following exposure to a testing substrate such as a carbon or nitrogen source, or an antimicrobial, the response of an organism may be detected. This detection may be visual (*i.e.*, by eye) or accomplished with the assistance of machine(s) (*e.g.*, the Biolog

MicroStation Reader™). For example, the response of organisms to carbon sources may be detected as turbidity in the suspension due to the utilization of the testing substrate by the organisms. Likewise, growth can be used as an indicator that an organism is not inhibited by certain antimicrobials. In one embodiment, color is used to indicate the presence or absence of organism growth/metabolism.

As used herein, the terms "chromogenic compound" and "chromogenic substrate," refer to any compound useful in detection systems by their light absorption or emission characteristics. ~~The term is intended to encompass any enzymatic cleavage products, soluble,~~ as well as insoluble, which are detectable either visually or with optical machinery. Included within the designation "chromogenic" are all enzymatic substrates which produce an end product which is detectable as a color change. This includes, but is not limited to any color, as used in the traditional sense of "colors," such as indigo, blue, red, yellow, green, orange, brown, etc., as well as fluorochromic or fluorogenic compounds, which produce colors detectable with fluorescence (*e.g.*, the yellow-green of fluorescein, the red of rhodamine, etc.). It is intended that such other indicators as dyes (*e.g.*, pH) and luminogenic compounds be encompassed within this definition.

As used herein, the commonly used meaning of the terms "pH indicator," "redox indicator," and "oxidation-reduction indicator," are intended. Thus, "pH indicator" encompasses all compounds commonly used for detection of pH changes, including, but not limited to phenol red, neutral red, bromthymol blue, bromcresol purple, bromcresol green, bromchlorophenol blue, m-cresol purple, thymol blue, bromcresol purple, xylenol blue, methyl red, methyl orange, and cresol red. The terms "redox indicator" and "oxidation-reduction indicator" encompass all compounds commonly used for detection of oxidation/reduction potentials (*i.e.*, "eH") including, but not limited to various types or forms of tetrazolium, resazurin, methylene blue, and quinone-imide redox dyes including the compounds known as "methyl purple" and derivatives of methyl purple. The quinone-imide redox dye known as methyl purple is referred to herein as "redox purple." In a particularly preferred embodiment, "redox purple" comprises the compound with the chemical structure shown in Figure 5, VI. It is contemplated that analogous derivatives of the reagent (*e.g.*, alkali salts, alkyl O-esters), with modified properties (*e.g.*, solubility, cell permeability, toxicity, and/or modified color(s)/absorption wavelengths) will be produced using slight modifications of the methods described in Example 13. It is also contemplated that various forms of redox purple (*e.g.*,

salts, etc.). may be effectively used in combination as a redox indicator in the present invention.

As used herein, the terms "testing means" and "testing device" are used in reference to testing systems in which at least one organism is tested for at least one characteristic, such as utilization of a particular carbon source, nitrogen source, or chromogenic substrate, and/or susceptibility to an antimicrobial agent. This definition is intended to encompass any suitable means to contain a reaction mixture, suspension, or test. It is intended that the term encompass microtiter plates, petri plates, microcard devices, or any other supporting structure that is suitable for use. For example, a microtiter plate having at least one gel-initiating agent included in each of a plurality of wells or compartments, comprises a testing means. Other examples of testing means include microtiter plates without gel-initiating means included in the well. It is also intended that other compounds such as carbon sources or antimicrobials will be included within the compartments. The definition is also intended to encompass a "microcard" or miniaturized plates or cards which are similar in function, but much smaller than standard microtiter plates (for example, many testing devices can be conveniently held in a user's hand). It is not intended that the present invention be limited to a particular size or configuration of testing device or testing means. For example, it is contemplated that various formats will be used with the present invention, including, but not limited to microtiter plates, microcards, petri plates, petri plates with internal dividers used to separate different media placed within the plate, test tubes, as well as many other formats.

As used herein, the term "gelling agent" is used in a broad generic sense, and includes compounds that are obtained from natural sources, as well as those that are prepared synthetically. As used herein, the term refers to any substance which becomes at least partially solidified when certain conditions are met. For example, one gelling agent encompassed within this definition is Gelrite™, a gellan which forms a gel upon exposure to divalent cations (e.g., Mg^{2+} or Ca^{2+}). Gelrite™ is produced by deacetylating a natural polysaccharide produced by *Pseudomonas elodea*, and is described by Kang *et al.* (U.S. Patent Nos. 4,326,052 and 4,326,053, herein incorporated by reference).

Included within the definition are various gelling agents obtained from natural sources, including protein-based as well as carbohydrate-based gelling agents. One example is bacteriological agar, a polysaccharide complex extracted from kelp. Also included within the definition are such compounds as gelatins (e.g., water-soluble mixtures of high molecular weight proteins obtained from collagen), pectin (e.g., polysaccharides obtained from plants).

carrageenans and alginic acids (*e.g.*, polysaccharides obtained from seaweed), and gum (*e.g.*, mucilaginous excretions from some plants and bacteria). It is contemplated that various carrageenan preparations will be used in the present invention, with iota carrageenan comprising a preferred embodiment. It is also contemplated that gelling agents used in the present invention may be obtained commercially from a supply company, such as Difco, BBL, Oxoid, Marcor, Sigma, or any other source.

It is not intended that the term "gelling agent" be limited to compounds which result in the formation of a hard gel substance. ~~A spectrum is contemplated, ranging from merely a~~ more thickened or viscous colloidal suspension to one that is a firm gel. It is also not intended that the present invention be limited to the time it takes for the suspension to gel.

Importantly, it is intended that the present invention provides a gelling agent suitable for production of a matrix in which organisms may grow (*i.e.*, a "gel matrix"). The gel matrix of the present invention is a colloidal-type suspension of organisms produced when organisms are mixed with an aqueous solution containing a gelling agent, and this suspension is exposed to a gel-initiating agent. It is intended that this colloidal-type gel suspension be a continuous matrix medium throughout which organisms may be evenly dispersed without settling out of the matrix due to the influence of gravity. The gel matrix must support the growth of organisms within, under, and on top of the gel suspension.

As used herein the term "gel-initiating agent" refers to any compound or element which results in the formation of a gel matrix, following exposure of a gelling agent to certain conditions or reagents. It is intended that "gel-initiating agent" encompass such reagents as cations (*e.g.*, Ca^{2+} , Mg^{2+} , and K^{+}). Until the gelling agent contacts at least one gel-initiating agent, any suspension containing the gelling agent remains "ungelled" (*i.e.*, there is no thickening, increased viscosity, nor hardening of the suspension). After contact, the suspension will become more viscous and may or may not form a rigid gel (*i.e.*, contact will produce "gelling").

As used herein, the term "inoculating suspension" or "inoculant" is used in reference to a suspension which may be inoculated with organisms to be tested. It is not intended that the term "inoculating suspension" be limited to a particular fluid or liquid substance. For example, inoculating suspensions may be comprised of water, saline, or an aqueous solution which includes at least one gelling agent. It is also contemplated that an inoculating suspension may include a component to which water, saline or any aqueous material is added. It is contemplated in one embodiment, that the component comprises at least one component

useful for the intended microorganism. It is not intended that the present invention be limited to a particular component.

As used herein, the term "kit" is used in reference to a combination of reagents and other materials. It is contemplated that the kit may include reagents such as carbon sources, nitrogen sources, chromogenic substrates, antimicrobials, diluents and other aqueous solutions, as well as microplates (*e.g.*, GN, GP, YT, SF-N, SF-P, and other MicroPlates™, obtained from Biolog), inoculants, microcards, and plated agar media. The present invention contemplates other reagents useful for the growth, identification and/or determination of the antimicrobial susceptibility of microorganisms. For example, the kit may include reagents for detecting the growth of microorganisms following inoculation of kit components (*e.g.*, tetrazolium or resazurin included in some embodiments of the present invention). It is not intended that the term "kit" be limited to a particular combination of reagents and/or other materials. Further, in contrast to methods and kits which involve inoculating organisms on or into a preformed matrix such as an agar surface or broth, the present invention involves inoculation of a testing plate in which the organisms are suspended within a gel-forming matrix.

As used herein, the term "primary isolation" refers to the process of culturing organisms directly from a sample. Thus, primary isolation involves such processes as inoculating an agar plate from a culture swab, urine sample, environmental sample, etc. Primary isolation may be accomplished using solid or semi-solid agar media, or in liquid. As used herein, the term "isolation" refers to any cultivation of organisms, whether it be primary isolation or any subsequent cultivation, including "passage" or "transfer" of stock cultures of organisms for maintenance and/or use.

As used herein, the term "presumptive diagnosis" refers to a preliminary diagnosis which gives some guidance to the treating physician as to the etiologic organism involved in the patient's disease. Presumptive diagnoses are often based on "presumptive identifications," which as used herein refer to the preliminary identification of a microorganism based on observation such as colony characteristics, growth on primary isolation media, gram stain results, etc.

As used herein, the term "definitive diagnosis" is used to refer to a final diagnosis in which the etiologic agent of the patient's disease has been identified. The term "definitive identification" is used in reference to the final identification of an organism to the genus and/or species level.

GENERAL DESCRIPTION OF THE INVENTION

The present invention is predicated in part on the discovery that various organisms may be identified and differentiated based on differential biochemical reactions observed in gelled media. The multiple test medium of the present invention permits presumptive and rapid microbiological screening of various specimens. In particular, this invention in the form of a kit, is suitable for the easy and rapid biochemical testing of various microorganisms, including the actinomycetes and fungi.

~~The present invention also contemplates a multitest indicator plate that is generally~~ useful in the identification and antimicrobial sensitivity testing of microorganisms. This medium and method are particularly targeted toward some of the most economically important organisms, as well as species of clinical importance. It is not intended that the invention be limited to a particular genus, species nor group of organisms.

It is contemplated that the present invention be used with various gelling agents, including, but not limited to agar, pectin, alginate, alginic acid, silica, gellans and gum. In one embodiment, the pectin medium of Roth (U.S. Patent Nos. 4,241,186, and 4,282,317; herein incorporated by reference) is used. However, this is not a preferred embodiment, as pectin is not a colorless compound itself. In one preferred embodiment, the gellan of Kang *et al.* (U.S. Patent Nos. 4,326,052 and 4,326,053, herein incorporated by reference) is used. In another preferred embodiment, carrageenan is used as the gelling agent. In a particularly preferred embodiment, carrageenan type II or any carrageenan which contains predominantly the iota form of carrageenan is used. In each embodiment, the microorganisms to be tested are mixed in a suspension comprising a gelling agent, and then inoculated into a well, compartment, or other receptacle, which contains the biochemical(s) to be tested, along with a gel-initiating agent such as various cations. Upon contact of the gelling agent with the gel-initiating agent (*e.g.*, cations), the suspension solidifies to form a viscous colloid or gel, with the organisms evenly distributed throughout.

The present invention contemplates a testing device that is a microplate similar in structure to microtiter plates ("microplates" or "MicroPlates™") commonly used in the art and commercially available from numerous scientific supply sources (*e.g.*, Biolog, Fisher, etc.). Thus, in one embodiment, standard 96-well microtiter plates are used.

An alternate embodiment of the invention generally relates to a "microcard" device for the multiparameter testing of chemical, biochemical, immunological, biomedical, or microbiological samples in liquid or liquid suspension form in a small, closed, easy-to-fill

device, and is particularly suitable for multiparameter testing and identification of microorganisms. It is not intended that the present invention be limited to a particular sized device. Rather, this definition is intended to encompass any device smaller than the commonly used, 96-well microplates. In one particularly preferred embodiment, the microcard is approximately 75 mm in width and 75 mm in length, and approximately 3 mm in depth. Approximately one-tenth the volume of cells are used to inoculate the compartments of the device, as compared to standard microplates. Indeed, the present invention contemplates a device comprising: a) a housing; b) a testing region contained within the housing; c) a liquid receiving means on an external surface of the housing; d) a liquid flow-directing means providing liquid communication between the testing region and the liquid receiving means; and e) a gas-venting, liquid barrier in fluidic communication with the testing region.

After the device has been filled, a non-venting, sealing tape can be applied to the device to cover the gas-venting, liquid barrier to reduce the evaporation of the liquid from the device; the tape can permit the molecular diffusion of oxygen into or out of the device to maintain the desired chemical or biochemical environment within the device for successful performance of the test. Where the liquid receiving means comprises liquid entry ports, a similar closing tape can be applied to close the port or ports to prevent spilling and evaporation of the liquid therefrom.

With any of the testing formats, the visual result that is detected by eye or by instrument can be any optically perceptible change such as a change in turbidity, a change in color, or the emission of light, such as by chemiluminescence, bioluminescence, or by Stokes shift. Color indicators may be, but are not limited to, redox indicators (*e.g.*, tetrazolium, resazurin, and/or redox purple), pH indicators, or various dyes and the like. Various dyes are described in U.S. Patents Nos. 4,129,483, 4,235,964 and 5,134,063 to Barry R. Bochner, hereby incorporated by reference. *See also* B. R. Bochner, *Nature* 339:157 (1989); and B.R. Bochner, *ASM News* 55:536 (1990). A generalized indicator useful for practice of the present invention is also described by Bochner and Savageau. *See* B. Bochner and M. Savageau, *Appl. Environ. Microbiol.*, 33:434 (1977).

Testing based on the redox technology is extremely easy and convenient to perform. A cell suspension is prepared and introduced into the testing compartments of the device. Each compartment is prefilled with a different substrate.

In a preferred embodiment, all wells are prefilled with test formula comprising a basal medium that provides nutrients for the microorganisms, and a color-change indicator, and each compartment is prefilled with a different carbon compound or "testing substrate," against which the microorganism is tested. "Basal medium," as used herein, refers to a medium which provides nutrients for the microorganisms, but does not contain sufficient concentrations of carbon compounds to trigger a color response from the indicator. "Carbon compound," "carbon source" and "testing substrate" are equivalent terms, and are used interchangeably herein to refer to a carbon chemical in sufficient concentration as to trigger a color response from the indicator when it is utilized (metabolized) by a microorganism (e.g., GN, GP, YT, and other MicroPlates™ commercially available from Biolog). In a particularly preferred embodiment, redox purple is used as a redox indicator in the present invention.

One of the principal uses of the present invention is as a method and device for simple testing and speciation of microorganisms. The present invention contemplates microbiological testing based on the redox technology discussed above wherein a sample of a pure culture of microorganism is removed from a culture medium on which it has been grown and suspended in saline or water at a desired density. This suspension is then introduced into the compartments of the testing device which have been prefilled with basal medium, indicator, and substrate chemicals. The method is extremely easy and convenient to perform, and, unlike other approaches, the method and device do not require skilled personnel and cumbersome equipment.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μ M (micromolar); N (Normal); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); °C (degrees Centigrade); TSA (trypticase soy agar); YME or YEME (Yeast extract-malt extract agar); EMB (eosin methylene blue medium); MacConkey (MacConkey medium); Redigel (RCR Scientific, Goshen, IN); Gelrite™ (Merck and Co., Rahway, NJ, available from Sigma); Remel. (Remel, Lenexa, KS);

Oxoid (Oxoid, Basingstoke, England); BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD); DIFCO (Difco Laboratories, Detroit, MI); U.S. Biochemical (U.S. Biochemical Corp., Cleveland, OH); Fisher (Fisher Scientific, Pittsburgh, PA); Sigma (Sigma Chemical Co., St. Louis, MO.); Biolog (Biolog, Inc., Hayward, CA); ATCC (American Type Culture Collection, Rockville, Maryland); CBS (Centraalbureau Voor Schimmelcultures, Delft, Netherlands); CCUG (Culture Collection of University of Gothenberg, Gothenberg, Sweden); GSU (Georgia State University, Atlanta, GA); NRRL (USDA Northern Regional Research Laboratory, Peoria, IL); and NCYC (National Collection of Yeast Cultures, Norwich, England); NCCLS (National Committee for Clinical Laboratory Standards); API (API Analytab Products, Plainview, NY); Flow (Flow Laboratories, McLean, VA); Biomerieux (Biomerieux, Hazelwood, MO).

The following Tables list the principal bacterial strains used in the following Examples, with Table 2 listing the various actinomycetes, and Table 3 listing other species of microorganisms.

TABLE 2
Actinomycetes Tested

Organism	Source and Number
<i>Actinomadura ferruginea</i>	USDA NRRL B-16096
<i>Actinoplanes rectilineatus</i>	USDA NRRL B-16090
<i>Micromonospora chalcea</i>	USDA NRRL B-2344
<i>Norcardiopsis dassonvillei</i>	USDA NRRL B-5397
<i>Saccharopolyspora hirsuta</i>	USDA NRRL B-5792
<i>Streptomyces albidoflavus</i>	USDA NRRL B-1271
<i>Streptomyces coeruleoribidus</i>	USDA NRRL B-2569
<i>Streptomyces griseus</i>	USDA NRRL B-2682
<i>Streptomyces hygroscopicus</i>	USDA NRRL B-1477
<i>Streptomyces lavendulae</i>	USDA NRRL B-1230
<i>Streptoverticillium salmonis</i>	USDA NRRL B-1484

TABLE 3
Other Organisms Tested

Organism	Source and Number
<i>Escherichia coli</i>	ATCC #25922
<i>Staphylococcus aureus</i>	ATCC #29213
<i>Providencia stuartii</i>	ATCC #33672
<i>Pseudomonas cepacia</i>	ATCC #25416
<i>Neisseria lactamica</i>	CCUG#796
<i>Xanthomonas maltophilia</i>	ATCC#13637
<i>Vibrio metschnikovii</i>	ATCC#7708
<i>Cedecea neteri</i>	ATCC#18763
<i>Rhodococcus equi</i>	ATCC#6939
<i>Dipodascus ovetensis</i>	ATCC #10678
<i>Cryptococcus laurentii</i>	CBS#139
<i>Cryptococcus terreus A</i>	CBS#1895
<i>Kluyveromyces marxianus</i>	GSU#C90006070
<i>Saccharomyces cerevisiae A</i>	NCYC##505
<i>Williopsis saturnus var. saturnus</i>	GSU#WC-37
<i>Penicillium notatum</i>	ATCC#9179
<i>Penicillium chrysogenum</i>	ATCC#11710
<i>Rhizomucor pusillus</i>	ATCC#32627
<i>Aspergillus niger</i>	ATCC#16404
<i>Tricophyton mentagrophytes</i>	ATCC#9129

EXAMPLE 1

Primary Growth of Actinomycetes

In this example, several attempts were made to grow various actinomycetes in R2A liquid media prepared from the recipe of Reasoner and Geldreich (Reasoner and Geldreich, Appl. Environ. Microbiol., 49:1-7 [1985]), prior to preparation of inoculum suspensions for inoculating commercially available MicroPlates™ from Biolog (Biolog's GN, GP, and YT MicroPlates™). This method proved unsuccessful and cumbersome. Also, it was virtually impossible to obtain uniform (homogenous) cultures of satisfactory quality.

Next, these organisms were grown on the surface of various agar media. It was thought this might provide a very simple means to harvest spores from the culture, as the colonies tend to anchor into the agar matrix itself. The media used in this example included Sporulation Agar (described by R. Atlas in Handbook of Microbiological Media, CRC Press, Boca Raton, FL, p. 834 [1993]), and YEME Agar with glucose omitted (described by E.B. Shirling and D. Gottlieb, in "Methods for Characterization of *Streptomyces* Species." Int'l J. System. Bacteriol., 16:313-330 [1966])(hereinafter referred to as YEMEWG).

Sporulation Agar (also known as m-Sporulation Agar) comprises agar (15 g/l), glucose (10 g/l), tryptose (2 g/l), yeast extract (1 g/l), beef extract (1 g/l), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1 $\mu\text{g/l}$), pH 7.2 ± 0.2 at 25°C . These ingredients are added to 1 liter of distilled/deionized water, and mixed thoroughly with heat to boiling. After the mixture has dissolved, it is autoclaved at 15 psi (121°C) for 15 minutes, and dispensed into plates.

YEMEWG Agar comprises Bacto yeast extract (4 g/l; Difco), and Bacto-malt extract (10 g/l; Difco). These ingredients are added to 1 liter of distilled/deionized water and mixed thoroughly. The pH is adjusted to 7.3, and agar (20 g/l) is added to the mixture. The mixture is then autoclaved at 121°C for 15-20 minutes, and dispensed into Petri plates after it is sufficiently cooled. YEMEWG was used because preliminary studies indicated that, while glucose-containing YEME agar was adequate for growth of the *Streptomyces* species, genera such as *Nocardiosis* and *Actinoplanes* grew better when glucose was omitted from the medium recipe.

Because of the interest in obtaining spores, media that encourage sporulation were tried. For example, YEMEWG was found to be particularly useful, as this medium gave satisfactory growth and sporulation of most strains tested within 2-4 days of incubation at 26°C . Various agar concentrations were tested during these preliminary studies, and it was further observed that when YEMEWG was used, improved sporulation occurred in the presence of a higher agar concentration (e.g., 25 g/l, rather than the 15 g/l, traditionally used in microbiological agar media).

This approach of growing actinomycetes on a sporulation-inducing medium would have the additional benefit of standardizing the physiological state of the organisms, and would permit preparation of inocula primarily from spheroidal spores. It was usually a relatively simple matter to produce uniform, homogeneous suspensions containing spores. Occasionally, however, large clumps of the organisms and their aerial mycelia are obtained which do not readily disperse in solution. When clumps are formed, the suspension is

allowed to sit for a few minutes, permitting the large fragments to settle to the bottom of the tube. Use of a light inoculum (*i.e.*, a 1:10 dilution of an initial suspension where the initial suspension has a transmittance level of 70%) also helps avoid problems with clumping of large fragments. Therefore, clumps can be avoided in the preparation of the final inoculum because only a small, clump-free aliquot of the initial suspension is used. For those organisms that sporulate poorly, fragments of rods and/or mycelial filaments were obtained from the agar surface in the same manner.

This example highlights the advantages of the present invention for the primary growth and subsequent characterization of actinomycetes, in contrast to references that indicate growth of actinomycetes is very slow. For example, Bergey's Manual® (T. Cross, "Growth and Examination of Actinomycetes--Some Guidelines," in J. Holt *et al.*, "The Actinomycetes," Bergey's Manual® of Determinative Bacteriology, 9th ed., Williams & Wilkins, Baltimore, pp. 605-609 [1994]) indicates that "mature aerial mycelium with spores may take 7-14 days to develop, and some very slow-growing strains may require up to 1 month's incubation." This is in stark contrast to the present invention, in which heavy growth and sporulation is achieved within 2-4 days of incubation.

EXAMPLE 2

Preparation of Inoculum

In this experiment, a method more optimal for preparation of a homogeneous inoculum was determined. For example, it was found that an easy and reproducible method was to grow the organisms as described in Example 1 on YEMEWG- prepared with 25 g/l agar, or other suitable agar medium. A low density inoculum (*i.e.*, 0.01 to 0.1 OD₅₉₀) was then prepared by moistening a cotton swab and rubbing it across the top of the colonies to harvest mycelia and spores. It was determined that sterilized water and 0.85% sterile saline worked reasonably well as a suspension medium for all strains. However, some strains exhibited a preference for one or the other. For example, *Streptomyces coeruleoribidus*, *S. hygroscopicus*, and *S. albidoflavus* produced an average of ten additional positive reactions when water was used as the suspension medium, whereas thirteen additional positive reactions were observed for *S. lavendulae* when saline was used as the suspension medium. The majority of the Actinomycetes performed better when water was used. Therefore, water was used routinely to prepare the suspensions.

EXAMPLE 3

Preparation of Multi-Test Plates

The inocula prepared as described in Example 2 were used to inoculate various Biolog MicroPlates™, including the commercially available GN, GP, and YT MicroPlates™. A few strains worked well upon inoculation into the GN or GP MicroPlates™ (e.g., *S. lavendulae*). However, for most strains (e.g., *A. ferruginea*, and *N. dassonvillei*) no positive reactions were observed. In addition, positive reactions were observed in all of the test wells for some organisms (e.g., *S. hirsuta*), indicating that there was a problem with false positive results.

Much improved results were obtained when the wells located in the bottom five rows of the YT MicroPlate™ were used. It was thought that this observation was due to the absence of tetrazolium in these wells, as the tetrazolium present in the other wells appeared to inhibit the growth of the organisms. This was confirmed by testing the ability of the organisms to grow on YEMEWG agar media containing various concentrations of tetrazolium (20, 40, 60 and 80 mg/l). Many strains (e.g., *S. coeruleoribidus*, *S. hygroscopicus*, *S. lavendulae*, *M. chalcea*, *N. dassonvillei*, and *A. rectilineatus*) were inhibited at all of these tetrazolium concentrations. Other organisms, such as *S. griseus*, *S. albidoflavus*, and *S. hirsuta*, were somewhat inhibited at the higher tetrazolium concentrations, but grew in tetrazolium concentrations of 20 and 40 mg/l.

Based on these experiments, MicroPlates™ were then tested that contained no tetrazolium (e.g., "SF-N" [GN MicroPlate™ without tetrazolium] and "SF-P" [GP MicroPlate™ without tetrazolium] MicroPlates™). These plates were inoculated with water or saline suspensions of various actinomycetes, and incubated at 26°C for 1-4 days. Increased turbidity (i.e., growth of the organisms) was readable visually, or with a microplate reader (e.g., a Biolog MicroStation Reader™, commercially available from Biolog), in as little as 24 hours for some strains. For the slow growing strains, growth was readable and the results interpretable within 3-4 days, representing a significant improvement over the 7-10 day incubation period required using routine methods.

EXAMPLE 4

Use Of Gelrite™

Although growth was observable in the multi-test system described in Example 3, the results were still not completely satisfactory, due to the unique growth characteristics of the actinomycetes. Many of these strains adhered to the plastic walls of the microplate wells, thereby making detection of increased turbidity less than optimal. When the inoculating suspension is a liquid, turbidity often was concentrated along the outer circumference of the wells, rather than producing a uniform dispersion of turbidity throughout the wells.

In order to facilitate uniform dispersion of the inoculating suspension containing organisms throughout the well, a gelling agent was added to the suspension to prevent individual cells from migrating to the well walls. For example, preparations of Gelrite™ (commercially available from Sigma, under this name, as well as "Phytigel") were found to be highly satisfactory. Gelrite™ does not form a gel matrix until it is exposed to gel-initiating agents, in particular, positively charged ions such as divalent cations (e.g., Mg^{2+} and Ca^{2+}). As soon as the Gelrite™ comes into contact with the salts present in the bottom of the microplate wells, the gelling reaction begins and results in the formation of a gel matrix within a few seconds.

Various concentrations of Gelrite™ were tested, including 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%. All concentrations gelled in the microplate, with the higher concentrations producing a harder gel.

In view of the fact that most of the actinomycetes are obligate aerobes, there was a concern that the oxygen concentration within the gel must be sufficient to permit growth. Thus, various gel depths were tested by using 50, 100, or 150 μ l suspensions of organisms in the wells. Each of these depths resulted in good growth of organisms, although it was observed that 0.4% Gelrite™ and an inoculum of 100 μ l produced optimal results, even with organisms such as *Streptomyces lavendulae*, a species that is strongly hydrophobic and clings to the walls of wells when it is suspended in water. The 0.4% concentration of Gelrite™ was found to produce an appropriate degree of viscosity to readily permit preparation of microbial suspensions and still be easily pipetted.

The entire procedure for growth and testing of the actinomycetes required a total of 3-7 days, including primary inoculation on YEMEWG medium and other suitable media to determination and analysis of the final results. Importantly, a minimum amount of personnel

time was required (i.e., just the few minutes necessary to inoculate the primary growth medium and then prepare the suspension for biochemical testing). Thus, the present invention provides a much improved means for the rapid and reliable identification of actinomycetes.

EXAMPLE 5

Comparison of Water and Gelrite™

In this Example, the eleven actinomycetes listed in Table 2 were tested in both water and gel suspensions. For each organism, a water suspension of organisms with an optical transmittance of 70%, was diluted 1:10 in either water or 0.4% Gelrite™. Thus, two samples of each organism were produced, one sample being a water suspension and one sample being a suspension which included Gelrite™.

One hundred microliters of each sample were inoculated into SF-P MicroPlates™ (GP MicroPlates™ without tetrazolium; commercially available from Biolog). The MicroPlates™ were incubated at 27°C for 48 hours, and observed for growth. As shown in the table below, the number of positive reactions increased dramatically for the organisms suspended in Gelrite™, as compared to water.

TABLE 4

Growth of Selected *Streptomyces* Species

	Number of Positive/ Borderline Reactions in Water Suspensions (+/b)	Number of Positive/Borderline Reactions in Gel Suspensions (+/b)
<i>Streptomyces coeruleorubidus</i>	5/35	35/25
<i>Streptomyces griseus</i>	30/15	43/12
<i>Streptomyces lavendulae</i>	8/18	24/12

EXAMPLE 6

Use of Resazurin

In this Example, three concentrations of resazurin dye (25 mg/l, 50 mg/l, and 75 mg/l) were used as a redox color indicator of organism growth and metabolism. All of the eleven actinomycete strains listed in Table 2 were tested using these three concentrations of resazurin, and 0.4% Gelrite™.

The expected color reaction, a change from blue to pink and eventually to colorless, as the dye is progressively reduced, occurred with all test organisms after 48 hours of incubation at 27°C. This observation provides a supplemental indicator of organism metabolism in addition to turbidity. No single resazurin concentration provided uniformly optimal results. For example, *N. dassonvillei* produced a good differential pattern of color change at 25 mg/l and 50 mg/l, whereas *S. lavendulae* produced false positive results (*i.e.*, all colorless wells) at the lower concentrations (25 mg/l and 50 mg/l), but a good differential pattern of color change at 75 mg/l.

Although the resazurin concentration may need to be adjusted depending upon the organism tested, the use of resazurin as a color indicator may provide additional valuable information to characterize organisms at the species or strain level.

In the course of these experiments, it was also observed that pigments produced by some actinomycetes in the various carbon sources tended to create very distinct and unique patterns. The unexpected observation was made that pigment production was enhanced by using a gel-forming substance in the inoculant.

Thus, different color patterns were obtained with the differing resazurin dye concentrations in combination with the natural pigments produced. For example, at 50 mg/l resazurin, *M. chalicea* produced a range of color intensities from colorless to light pink to bright pink and purple. *S. hygroscopicus* produced a range of colors from yellow and orange, to colorless, pink and blue. Other species exhibited other distinct color patterns in the wells. This additional information related to pigmentation and resazurin dye reduction, may be valuable to taxonomists and others interested in characterizing specific strains and/or species of actinomycetes.

EXAMPLE 7

Use of Alternative Gelling Agents

Other gelling agents were tested in this Example. In addition to Gelrite™, alginic acid, carrageenan type I, carrageenan type II, and pectin were tested for their suitability in the present invention. All of these compounds are commercially available from Sigma.

Of these compounds, pectin was found to be unsuitable when tested by adding 1% pectin to SF-P MicroPlates™. Pectin has a yellowish cast to it, and is therefore not a colorless or clear compound. Furthermore, gelling was dependent upon the presence of sugars in the microplate wells. Because many of the substrates tested in this multitest format do not contain sugars, gelling did not occur uniformly in all wells.

All of these gelling agents with the exception of pectin, were tested with the eleven actinomycetes listed in Table 2. The same MicroPlates™ (SF-P), incubation time and temperature, as described in Example 5 above, were used. The only variables were the different gelling agents and varying concentrations of these agents.

The optimal viscosity and performance for each gelling agent was determined. Optimal viscosity and performance was achieved at 1% alginic acid; 0.2% was optimum for both types of carrageenan; and 0.4% was optimum for Gelrite™. All of these gelling agents were also diluted to half the above concentrations and found to be useful even at these lower concentrations.

Overall, the results for Gelrite™ and carrageenan types I and II were similar, and the difference in gel concentration did not affect the results significantly. However, the results for alginic acid were not as clearcut when the MicroPlates™ were observed by eye, as compared to the use of an automatic plate reader (e.g., Biolog MicroStation Reader™, Biolog). Indeed, when read by eye, the results with alginic acid were somewhat inferior to those obtained with Gelrite™. Carrageenan type II was slightly better than type I and it was also comparable to or better than Gelrite™. Surprisingly, the carrageenan type II functions as effectively as the Gelrite™, although the carrageenan does not form a rigid gel. This indicates that it is not necessary that a rigid gel be formed in order for the beneficial effects of these colloidal gelling agents to be observed.

EXAMPLE 8

Testing of Other Bacterial Species

In addition to the actinomycetes, the present invention is also suitable for the rapid characterization of numerous and diverse organisms, such as those listed in Table 3. The gram-negative bacteria tested covered a range of genera and tribes, including *Pseudomonas cepacia*, *Providencia stuartii*, *Neisseria lactamica*, *Xanthomonas maltophilia*, *Vibrio metschnikovii*, *Cedecea neteri*, and *Escherichia coli*. Various gram-positive bacteria were also tested, including *Rhodococcus equi* and *Staphylococcus aureus*.

These organisms were tested basically as described in Example 5 above, with GN MicroPlates™ (Biolog) used to test the gram-negative organisms, and GP MicroPlates™ (Biolog) used to test the gram-positive organisms. In addition, ES MicroPlates™ (Biolog) were also tested with some of the gram-negative species. Inoculation in 0.4% Gelrite™ was compared to inoculation in 0.85% saline. The inoculation densities used were those normally recommended for these MicroPlate™ test kits (55% transmittance for the gram-negative organisms, and 40% for the gram-positive organisms). Following inoculation of the MicroPlates™ with 150 µl suspensions of organisms in either saline or Gelrite™ per well, the MicroPlates™ were incubated at 35°C for 16-24 hours.

All of these organisms performed well in the gel, with most producing better results in gel than in saline. For example, in the ES MicroPlate™, *E. coli* produced 43 positive reactions within 24 hours when the gel was used, but only 36 positive reactions when saline was used. A correct identification of *C. neteri* was obtained after only 4 hours of incubation in the Gelrite™, whereas overnight incubation was required for saline. Thus, a correct identification of this organism is possible in a much shorter time period than the 24 hour incubation usually required for traditional testing methods.

In contrast to conventional biochemical testing materials and methods traditionally used, the present invention often achieves a definitive identification in a significantly shorter time period.

EXAMPLE 9

Testing of Eukaryotic Microorganisms--Yeasts

5 This experiment was designed to determine the suitability of the present invention for use in identification of eukaryotic microorganisms, such as yeasts. In this experiment, two types of reactions were observed to establish a metabolic pattern: a) assimilation reaction tests which are based on turbidity increases due to carbon utilization by the organisms; and b) oxidation tests, which also test for carbon utilization, but which detect utilization via a redox color change of the organism suspension.

10 In this experiment, yeasts were first grown on BUY Agar (Biolog) a solid agar medium, and harvested from the agar surface as described in Example 2 above. The organisms included in this example are listed in Table 3 (*D. ovetensis*, *C. laurentii*, *C. terreus*, *K. marxianus*, *S. cerevisiae*, and *W. saturnus*). Biolog YT MicroPlates™ (available commercially from Biolog) were then inoculated with an inoculum having an optical
15 transmittance of 50%, in either water or 0.4% Gelrite™. Each well of the YT MicroPlate™ was inoculated with 100 µl of either the water or 0.4% Gelrite™ suspension of organisms. Thus, there were two sets of 6 MicroPlates™ each. The inoculated MicroPlates™ were incubated at 27°C, and the results observed at 24, 48, and 72 hours of incubation.

20 With the oxidation tests, in most cases, the color changes developed more rapidly in the plates with Gelrite™ used as the inoculant, compared to the plates with water as the inoculant. For example, *D. ovetensis*, *W. saturnus*, *K. marxianus*, and *C. laurentii* gave stronger reactions at 48 hours with Gelrite™. In contrast, *S. cerevisiae* and *C. terreus* gave stronger reactions at 48 hours with water.

25 With the assimilation tests, in all cases the Gelrite™ was superior or equivalent to the water inoculant. The data shown in the Tables below clearly demonstrate that more positive (+) and borderline (b) reactions were obtained overall, when Gelrite™ was used.

TABLE 5
Positive (+) and Borderline (b) Reactions
After One Day of Incubation

Organism	Water (+/b)	Gelrite™ (+/b)
<i>D. ovetensis</i>	0/5	17/7
<i>K. marxianus</i>	14/3	16/9
<i>W. saturnus</i>	9/7	40/9
<i>C. terreus A</i>	4/14	33/3
<i>C. laurentii</i>	61/5	67/8
<i>S. cerevisiae A</i>	24/5	22/2

TABLE 6
Positive (+) and Borderline (b) Reactions
After Two Days of Incubation

Organism	Water (+/b)	Gelrite™ (+/b)
<i>D. ovetensis</i>	9/2	22/2
<i>K. marxianus</i>	14/5	39/4
<i>W. saturnus</i>	23/7	46/5
<i>C. terreus A</i>	21/7	45/4
<i>C. laurentii</i>	65/0	77/3
<i>S. cerevisiae A</i>	24/6	24/0

TABLE 7
Positive (+) and Borderline (b) Reactions
After Three Days of Incubation

Organism	Water (+/b)	Gelrite™ (+/b)
<i>D. ovetensis</i>	21/9	23/7
<i>K. marxianus</i>	27/5	43/7
<i>W. saturnus</i>	48/6	52/3
<i>C. terreus A</i>	20/8	58/5
<i>C. laurentii</i>	68/6	78/5
<i>S. cerevisiae A</i>	24/8	24/2

In these experiments, the surprising observation was made that some organisms could be identified faster due to better growth (*i.e.*, growth that appeared much more rapidly and at a greater density), in the plate with the Gelrite™, as compared to the plate with water. For example, *Dipodascus ovetensis* developed a metabolic reaction pattern sufficient for correct identification after 24 hours of incubation in the Gelrite™ plate, while 48 hours of incubation was required to make the proper identification in the water plate.

In addition, many of the limitations and deficiencies of currently commercially available yeast identification systems, such as the Minitek (BBL), API 20C (API), expanded Uni-Yeast-Tek System (Flow), and Vitek (Biomerieux) were overcome or avoided in the present example (*see e.g.*, G.A. Land (ed.), "Mycology," in H.D. Isenberg (ed.), Clinical Microbiology Procedures Handbook, American Society for Microbiology, in particular "Commercial Yeast Identification Systems," pp. 6.10.1 through 6.10.5, [1994]). For example, in the Vitek system, heavily encapsulated yeasts and isolates with extensive mycelial growth are sometimes difficult to suspend. As indicated above, this limitation is avoided by the present invention, allowing for reliable and reproducible testing procedures and systems. In summary, the Gelrite™ was shown to be clearly superior to water for the rapid identification of eukaryotic microorganisms.

EXAMPLE 11

Testing of Eukaryotic Microorganisms--Molds

This experiment was designed to determine the suitability of the present invention for use in identification of eukaryotic microorganisms, such as molds.

In this experiment, the molds were first grown on modified Sabouraud-Dextrose agar (commercially available from various sources, including Difco). This medium is prepared by thoroughly mixing dextrose (20 g/l), agar (20 g/l), and neopeptone (1 g/l) in 1 liter of distilled/deionized water. Heat is applied, until the mixture boils. The medium is autoclaved for 15 minutes at 15 psi (121°C). After cooling, the medium is distributed into petri plates.

The organisms included in this example are listed in Table 3 (*P. notatum*, *P. chrysogenum*, *R. pusillus*, *A. niger* and *T. mentagrophytes*). After they were grown on Sabouraud-Glucose agar, an inoculum was prepared as described in Example 1. YT and SP-F MicroPlates™ (Biolog) were then inoculated with a 1:10 dilution of a starting inoculum having an optical transmittance of 70%, in water, 0.2% carrageenan type II, or 0.4% Gelrite™.

Each well of the SF-P MicroPlates™ was inoculated with 100 µl of organisms suspended in either water, 0.2% carrageenan type II, or 0.4% Gelrite™. For the YT plates, 100 µl of organisms suspended in either water, or 0.4% Gelrite™ were used to inoculate the wells. The inoculated MicroPlates™ were incubated at 25°C, and the results observed by eye and using a MicroStation Reader™ (Biolog) at 24 hour increments for a total of 4 days of incubation.

In nearly all cases, the turbidity changes developed more rapidly in the plates with carrageenan or Gelrite™ used as the inoculant, compared to the plates with water as the inoculant. The data shown in the Tables below clearly demonstrate that for most organisms, more positive (+) and borderline (b) reactions were obtained overall, when carrageenan or Gelrite™ was used, as compared to water. The results in these Tables are those observed with the MicroStation Reader™ (Biolog).

It was also observed that the improvement in the results using Gelrite™ or carrageenan as the gelling agent were sometimes more apparent when the test results were read visually, rather than by a machine (Biolog's MicroStation Reader™). This was the case with *T. mentagrophytes*, where the improved results obtained with carrageenan were in fact, also obtained with Gelrite™, although the reader did not detect this accurately at 72 hours.

However, with longer incubation periods (e.g., 4-5 days), the visual and machine readings agree very well in nearly all cases.

TABLE 8

Positive(+)/Borderline (b) Reactions

After 72 Hours of Incubation in SF-P MicroPlates™

Organism	Carrageenan (+/b)	Gelrite™ (+/b)	Water (+/b)
<i>P. notatum</i>	54/11	52/14	47/11
<i>P. chrysogenum</i>	56/13	54/11	50/17
<i>R. pusillus</i>	4/13	5/5	2/6
<i>A. niger</i>	23/17	29/12	17/10
<i>T. mentagrophytes</i>	16/12	3/6	5/1

TABLE 9

Positive(+)/Borderline(b) Reactions

After 72 Hours of Incubation in YT MicroPlates™

Organism	Gelrite™ (+/b)	Water (+/b)
<i>P. notatum</i>	78/5	67/4
<i>P. chrysogenum</i>	81/1	75/10
<i>R. pusillus</i>	17/22	13/26
<i>A. niger</i>	78/2	51/11
<i>T. mentagrophytes</i>	2/1	2/1

EXAMPLE 12

Antimicrobial Susceptibility Testing

In this Example, the suitability of a gel matrix for use in antimicrobial susceptibility testing was investigated. Two organisms, *Staphylococcus aureus* (ATCC #29213) and *Escherichia coli* (ATCC#25922) were tested against a panel of three antimicrobial agents: ampicillin, kanamycin, and tetracycline. All three antimicrobials were obtained from Sigma. Biolog's MT MicroPlates™ (Biolog), were used with 12.5 µl of a 10% glucose solution added to each well. Kanamycin and tetracycline were dissolved in sterile water. Ampicillin was dissolved in phosphate buffer (pH 8.0)(0.1 M/l NaH₂PO₄·H₂O). For each antimicrobial agent, a dilution series ranging from 0.25 µg/ml to 32 µg/ml final concentration, was prepared. A 15 µl aliquot of each dilution was pipetted into the wells of the MicroPlates™, with water used to dilute the kanamycin and tetracycline, and phosphate buffer (pH 6.0)(0.1 M/l NaH₂PO₄·H₂O) used to dilute the ampicillin. For each MicroPlate™, a row of eight wells without antimicrobials was used as a control. In the MT MicroPlates™, tetrazolium is included as a color indicator. Unlike the actinomycetes, the most commonly isolated gram-negative and gram-positive bacteria are not significantly inhibited by the presence of tetrazolium in these MicroPlates™.

In addition to the MT MicroPlates™, Biolog's SF-N MicroPlates™ (GN MicroPlates™ without tetrazolium), and SF-P MicroPlates™ (GP MicroPlates™ without tetrazolium) were tested (all of these plates were obtained from Biolog). *E. coli* was inoculated into the SF-N MicroPlates™, and *S. aureus* was inoculated into the SF-P MicroPlates™. In these MicroPlates™, 25 mg/l of resazurin was added as a color indicator as an alternative to tetrazolium. In addition, 12.5 µl of 10% glucose solution and 15 µl of each antimicrobial dilution were added to each well, as described in the paragraph above.

All of the wells in all of the MicroPlates™ were inoculated with 100 µl of a very light suspension (e.g., a 1:100 dilution of a 55% transmittance suspension of *E. coli*, and a 1:100 dilution of a 40% transmittance suspension of *S. aureus*), and incubated overnight at 35°C.

For each organism and each MicroPlate™, 0.85% saline and 0.4% Gelrite™ were compared, by looking visually for the lowest antimicrobial concentration that inhibited dye (tetrazolium or resazurin) reduction. The minimum inhibitory concentration (MIC) for each organism was determined after 18 hours of incubation at 35°C. The MIC values for each organism, as determined from these experiments, are provided in the Tables below.

Table 10
MIC Determinations for *E. coli*
in MT MicroPlates™ Containing Tetrazolium
and SF-N MicroPlates™ Containing Resazurin

Diluent	Antimicrobial		
	Ampicillin	Kanamycin	Tetracycline
Saline	1-2	16-32	0.5-1
Gelrite™	2-4	8-16	0.5-1
NCCLS Expected Result	2-8	1-4	1-4

Table 11
MIC Determinations for *S. aureus*
in SF-P MicroPlates™ Containing Resazurin

Diluent	Antimicrobial		
	Ampicillin	Kanamycin	Tetracycline
Saline	1-4	16-32	0.25-2
Gelrite™	1-2	16-32	0.25-1
NCCLS Expected Results	0.25-1	1-4	0.25-1

As shown in these tables, the results in the Gelrite™ agreed with the results obtained with saline as an inoculant within one two-fold dilution. This is considered satisfactory according to the National Committee on Clinical Laboratory Standards (NCCLS) guidelines (see e.g., J. Hindler (ed.), "Antimicrobial Susceptibility Testing," in H.D. Isenberg (ed.), Clinical Microbiology Procedures Handbook, American Society for Microbiology, pp. 5.0.1 through 5.25.1, [1994]). In one instance, the MIC was slightly lower in saline as compared to Gelrite™. In three instances, the MIC's were slightly lower in Gelrite™, than in saline. Thus, the present invention provides a novel and useful alternative method for determination

of antimicrobial sensitivities of microorganisms. Another advantage of this invention is that the test may be conducted in a format that cannot be accidentally spilled.

EXAMPLE 13

Synthesis of Redox Purple

In this Example, the redox indicator referred to as "Redox Purple" was synthesized for use in the present invention. In this Example, the method of Graan *et al.* (T. Graan, *et al.*, "Methyl Purple, an Exceptionally Sensitive Monitor of Chloroplast Photosystem I Turnover: Physical Properties and Synthesis." *Anal Biochem.* 144:193-198 [1985]) was used with modifications. This synthesis is shown schematically in Figure 5 and the Roman numerals (*i.e.* I,II,III,IV and V) used in this Example refer to those shown in Figure 5. Unless otherwise indicated, the chemicals used in this Example were obtained from commercial sources such as Sigma.

Briefly, the benzoquinone-4-chloroimide (Figure 5, II) was produced by dissolving 5 g 4-aminophenol (Figure 5, I) in 1 N aqueous HCl (75 mL) (0°C), followed by the addition of 200 mL sodium hypochlorite (NaClO, 5% w/v) to produce a chloroimide derivative shown in Figure 5, Panel A. In this reaction, the solution was continuously stirred and the temperature maintained below 4°C during addition of the sodium hypochlorite. After stirring at room temperature for 12 hours, the yellow to orange colored product was isolated by filtration, washed with cold distilled water and dried in air and *in vacuo*. In this step, the product was vacuum filtered using a Buchner funnel, washed with a minimal amount of ice-cold water (approximately 30 ml) in the funnel, dried in air for approximately 24 hours, and dried overnight in a vacuum dessicator.

The synthesis of 1-(3-hydroxyphenyl)-ethanol (Figure 5, IV) was performed immediately prior to its use, by the reduction of 5 g 1-(3-hydroxyphenyl)-ethanone (available as m-hydroxyacetophenone from Tokyo Kasei kogyo Co., Ltd. Fukaya, Japan, with TCI America, in Portland, Oregon, being the U.S. distributor) (Figure 5, III) in water (300 mL) with sodium borohydride (NaBH₄, 1.5 g), as shown in Figure 5, Panel B. The reaction was warmed as necessary to dissolve the starting material and stirred until the evolution of H₂ ceased (approximately 1 hour). The pH was decreased to 2.0 (*i.e.*, with concentrated HCl) to remove excess borohydride, followed by addition of 150 ml saturated sodium borate.

The synthesis of redox purple was initiated by addition of the chloroimide derivative (II) to the freshly prepared solution of 1-(3-hydroxyphenyl)-ethanol (IV), in borate buffer ($\text{Na}_2\text{B}_4\text{O}_7/\text{H}_3\text{BO}_3$). Sodium arsenite (NaAsO_2 , 10 g) (Sigma) was added to the reaction solution, in order to promote the formation of the indophenol, as well as minimize the occurrence of side reactions. This reaction solution was stirred at room temperature for 2 hours, during which the blue color of the indophenol (Figure 5, V) appeared. The reaction mixture was then allowed to sit at room temperature for 7-8 days, during which the closure of the heterocyclic ring was allowed to occur due to formation of an oxymethylene group bridge between the two phenolic residues of the quinone-imide. The ring closure was accompanied by a change in the solution color to a dark purple.

The reaction mixture was filtered and the precipitate washed with minimal cold water as described above. The filtrate was saturated with an excess of solid sodium chloride (approximately 100 g), the solution was decanted off the excess salt on the bottom of the container, and the solution extracted with diethylether (5 X 100 mL) until no more orange-colored material was removed from the aqueous phase. Vigorous shaking of the ether and aqueous phases was avoided, as this was found in some experiments to result in formation of an intractable emulsion. The combined ether layers were back-extracted with 70 mM aqueous sodium carbonate solution (25 mL), the pH of the sodium carbonate solution reduced to 4.5 with glacial acetic acid, and the resulting mixture refrigerated overnight at 4°C. The redox purple precipitated as the free acid. Additional redox purple was obtained by acidifying the original aqueous phases with glacial acetic acid (pH 4.5) and repeating the above purification. The total yield obtained by this synthesis method was approximately 25%.

The purity of the redox purple synthesized according to this method was 95-98%, as determined by thin-layer chromatography, a method that is well known in the art (A. Braithwaite and F.J. Smith, in "Chromatographic Methods" Chapman and Hall [eds.], London [1985], pp. 24-50.). It was found that the redox purple compound was not very soluble in water as the free acid, but was quite soluble in slightly basic solutions (e.g., 1 N NaHCO_3), or in organic solvents (e.g. methanol, ethanol, dimethyl sulfoxide [DMSO], dimethyl formamide [DMF], etc.). The compound was observed to be a deep purple color (i.e., of approximately 590 nm as an absorption wavelength) in basic solution and an orange-red color (470 nm) in acidic solution. It is contemplated that analogous derivatives of the reagent (e.g., alkali salts, alkyl O-esters), with modified properties (e.g., solubility, cell permeability, toxicity, and/or modified color(s)/absorption wavelengths) will be produced using slight modifications of the

methods described here. It is also contemplated that various forms of redox purple (*e.g.*, salts, etc.), may be effectively used in combination as a redox indicator in the present invention.

EXAMPLE 14

Redox Purple and *E. coli* Identification

In this Example, redox purple was used as the redox indicator in the test system. *E. coli* 287 (ATCC #11775) was cultured overnight at 35°C, on TSA medium supplemented with 5% sheep blood. A sterile, moistened, cotton swab was used to harvest colonies from the agar plate and prepare six identical suspensions of organisms in glass tubes containing 18 ml of 0.85% NaCl, or 0.2% carrageenan type II. The cell density was determined to be 53-59% transmittance. One saline and one carrageenan suspension were used to inoculate Biolog GN Microplates™, with 150 µl aliquots placed into each well. The wells of this plate contain tetrazolium violet as the redox indicator. Two ml of a 2 mM solution of redox purple (sodium salt)(prepared as described in Example 13), or two ml of a 2 mM solution of resazurin (sodium salt) were added to the other tubes, to produce a final dye concentration of 200 µM. These suspensions were used to inoculate Biolog SF-N Microplates™. As with the GN Microplates™, aliquots of 150 µl were added to each well in the plates. The SF-N Microplates™ are identical to the GN MicroPlates™, with the exception being the omission of tetrazolium violet from the wells of the SF-N plates. The inoculated plates were incubated at 35°C for approximately 16 hours. The plates were then observed and the colors of the well contents recorded.

For the 0.85% NaCl and 0.2% carrageenan suspensions inoculated into the SF-N Microplate™, positive results were obtained for all three redox indicators (*i.e.*, redox purple, tetrazolium violet, and resazurin) in wells containing the following carbon sources: dextrin, tween-40, tween-80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arabinose, D-fructose, L-fucose, D-galactose, α-D-glucose, α -D-lactose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, L-rhamnose, D-sorbitol, D-trehalose, methyl pyruvate, mono-methyl succinate, acetic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, α-ketobutyric acid, D,L-lactic acid, propionic acid, succinic acid, bromosuccinic acid, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, D-serine, L-serine, inosine,

uridine, thymidine, glycerol, D,L- α -glycerol phosphate, glucose-1-phosphate, and glucose-6-phosphate.

For the 0.85% NaCl and 0.2% carrageenan suspensions, negative results were obtained for all three redox indicators (*i.e.*, redox purple, tetrazolium violet, and resazurin) in wells containing the following carbon sources: α -cyclodextrin, adonitol, D-arabitol, cellobiose, i-erythritol, xylitol, citric acid, D-glucosaminic acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α -ketovaleric acid, malonic acid, quinic acid, sebacic acid, L-histidine, hydroxy L-proline, L-leucine, and D,L-carnitine. The negative control wells containing water, instead of a carbon source were also negative for all three redox indicators.

For glycogen, D-psicose, succinamic acid, and glucuronamide, negative results were obtained with both the 0.85% NaCl and carrageenan suspensions with redox purple. However, positive results were obtained for both suspensions with tetrazolium violet and resazurin.

For gentiobiose, m-inositol, cis-aconitic acid, L-phenylalanine, L-pyroglutamic acid, phenylethylamine, putrescine, 2-amino ethanol, and 2,3-butanediol negative results were obtained with both the 0.85% NaCl and carrageenan suspensions with redox purple and tetrazolium violet. However, positive/negative results were obtained with the 0.2% carrageenan-suspension in resazurin; while the resazurin result with the 0.85% NaCl was negative.

For lactulose, D-raffinose, formic acid, α -hydroxybutyric acid, L-glutamic acid, and L-proline, negative results were observed with the 0.85% NaCl suspension tested with redox purple, although the remaining results were positive.

For sucrose and L-ornithine, negative results were obtained for both the 0.85% NaCl and 0.2% carrageenan suspensions tested with redox purple and tetrazolium violet. However, a negative result was observed for the 0.85% NaCl suspension tested with resazurin and a positive result was observed for the 0.2% carrageenan suspension.

For turanose, both the 0.85% NaCl and 0.2% carrageenan suspensions were negative when tested with redox purple, while the results for both tested with tetrazolium violet were equivocal (+/-), the result for the 0.85% NaCl suspension tested with resazurin was also equivocal (+/-), and the result for the 0.2% carrageenan tested with resazurin was positive.

For α -ketoglutaric acid, negative results were observed for both the 0.85% NaCl and 0.2% carrageenan suspensions tested with redox purple and tetrazolium violet, while positive results were observed for both suspensions tested with resazurin.

For D-saccharic acid, negative results were observed for both the 0.85% and 0.2% carrageenan suspensions tested with redox purple, while the result with tetrazolium violet was equivocal (+/-) for 0.85% NaCl and negative for carrageenan, and the result with resazurin was negative for the 0.85% NaCl and positive for 0.2% carrageenan suspensions.

For L-threonine, equivocal (+/-) results were observed for 0.2% carrageenan suspensions tested with redox purple and tetrazolium violet, while the result with resazurin was positive. For the 0.85% NaCl suspension, the result was negative for redox purple, and positive for tetrazolium violet and resazurin.

For γ -aminobutyric acid and urocanic acid, negative results were observed for both the 0.85% NaCl and 0.2% carrageenan suspensions tested with redox purple and tetrazolium violet, while equivocal (+/-) results were observed with 0.85% NaCl, and positive results were observed with the 0.2% carrageenan.

In the inoculated GN Microplate™ (containing tetrazolium violet), the wells corresponding to the carbon sources utilized by *E. coli* 287 became either a light or dark purple, while the wells corresponding to the carbon sources not utilized by this organism remained colorless. In contrast, in the inoculated SF-N Microplate™ (containing redox purple), the color pattern was virtually reversed. For negative wells with redox purple, a blue to purple (*i.e.*, blue-purple, purple-tinged blue, or violet) color was observed. In the SF-N Microplate™ plate, the wells corresponding to carbon sources utilized by this organism were light blue or were colorless, while the wells containing carbon sources not utilized by this organism remained dark blue. The color patterns were easily read and analyzed. Thus, the redox purple was shown to work in a manner that appears to be equivalent to tetrazolium violet for detecting carbon source utilization by bacteria. However, there were three colors observed with the plates which included resazurin (*i.e.*, blue, pink and colorless), making the redox purple a more useful redox indicator, as there was less ambiguity in the reading of the results.

The observation that none of the wells with redox purple was orange was very surprising, as the literature describing this compound indicated that there was an intermediate stage in the reduction of the dye which was expected to be reduced through the color

progression of blue to orange to colorless. This two-stage reduction is in contrast to the typical reaction observed with resazurin, which gives blue, pink, and colorless wells when analyzed in a like manner. The side-by-side data for the resazurin in this experiment, as well as other tests, confirms that it does form three colors. The degree to which the results of the various plates were in agreement are shown in the following Table.

**Table 12. Comparison of Redox Purple and Resazurin
with Tetrazolium Violet**

Solution	Dyes Compared	Number of Wells With Same Result (96 Wells/Plate)	% Agreement
Saline	Redox Purple/ Tetrazolium Violet	85/96	88.5
Gel	Redox Purple/ Tetrazolium Violet	92/96	95.8
Saline	Resazurin/ Tetrazolium Violet	95/96	99.0
Gel	Resazurin/ Tetrazolium Violet	91/96	94.8

The oxidized form of redox purple spectrally matches the reduced form of tetrazolium violet (*i.e.*, with a maximum absorbance at 590 nm). This may provide an advantage, as detection methods such as spectrophotometry settings may be used interchangeably with tetrazolium violet and redox purple.

EXAMPLE 15

Redox Purple and Identification of Fungi

In this Example, *Aspergillus niger*, *Penicillium chrysogenum*, and *Trichoderma harzianum* were tested using the redox purple redox indicator.

First, the above named organisms were tested using the GN Microplate. However, none of these organisms reduced the tetrazolium violet in the wells of the plate. Thus, redox purple was investigated for use as an alternative dye.

T. harzianum DAOM 190830 was cultured for seven days at 26°C on malt extract agar (Difco). A sterile, moistened cotton swab was used to harvest conidia from the culture and prepare a suspension in 16 ml of 0.25% Gelrite™. The cell density was determined to be

75% transmittance. A 2 ml aliquot of a 2 mM solution of redox purple was added to the suspension, along with 2 ml of 1 M triethanolamine-SO₄, pH 7.3. The final concentration of redox purple was 200 µM, and the final concentration of triethanolamine-SO₄ was 100 mM. The final suspension was mixed well and used to inoculate the wells of a Biolog SF-N Microplate™. In this Example, 100 µl of the suspension was added to each well. The inoculated SF-N Microplate™ was incubated at 30°C for approximately 24 hours, and observed.

For each carbon source utilized by the organism, the content of the wells was colorless. For each carbon source not utilized by the organism, the content of the wells was blue. In this Example, for this culture, positive results were obtained in the wells containing dextrin, glycogen, tween-40, tween-80, N-acetyl-D-glucosamine, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-sorbitol, D-trehalose, methyl pyruvate, mono-methyl succinate, citric acid, D-galacturonic acid, β-hydroxybutyric acid, α-ketoglutaric acid, quinic acid, sebacic acid, succinic acid, bromo succinic acid, succinamic acid, L-alanine, L-alanyl-glycine, L-asparagine, L-glutamic acid, γ-glutamyl-L-glutamic acid, L-ornithine, L-phenylalanine, L-proline, L-pyroglyutamic acid, L-serine, γ-amino butyric acid, inosine, and glycerol.

From the above Examples, it is clear that the present invention represents an unexpected and much improved system for the rapid biochemical testing of microorganisms, in many uses and formats (or configurations) and in particular, provides a major advance in the testing of actinomycetales and other spore-forming microorganisms. The results are highly surprising in view of the obligate aerobic nature of most of these organisms. Using the novel approach of embedding the organisms in a gel matrix, the biochemical test reactions are dispersed uniformly throughout the testing well, providing an easy to read indicator of organism growth and metabolism. In addition, both automated and manual systems may be used in conjunction with the present invention. For example, the results may be observed visually (*i.e.*, by eye) by the person conducting the test, without assistance from a machine. Alternatively, the results may be obtained with the use of equipment (*e.g.*, a microplate reader) that measures transmittance, absorbance, or reflectance through, in, or from each well of a multitest device such as microplate or microcard.

In summary, the present multitest gel-matrix invention provides numerous advances and advantages over the prior art, including: (1) much greater safety, as there is no spillage, nor aerosolization of cells, mycelia, nor spores, while performing or inoculating test wells; (2) faster biochemical reactions are produced, giving final results hours or days earlier than commonly used methods; (3) more positive biochemical reactions are obtained, giving a truer picture of the microorganisms' metabolic characteristics; (4) darker, more clear-cut biochemical reactions and color changes are obtained; (5) more uniform color and/or turbidity are obtained, as the cells, mycelia, and/or spores do not settle and clump together at the bottom of the wells, nor do they adhere to the sides of the wells; (6) the reactions are much easier to observe visually or with optical instruments (e.g., the Biolog MicroStation Reader™); and (7) the overall process for performing multiple tests is extremely simple and efficient, requiring very little labor on the part of the microbiologist. All of these advantages enhance the speed and accuracy of scoring test results in studies to characterize and/or identify microorganisms.

CLAIMS

What is claimed is:

1. A method for testing microorganisms comprising the steps of:
 - 5 a) providing a testing means comprising redox purple and one or more test substrates;
 - b) introducing microorganisms into said testing means; and
 - c) detecting the response of said microorganism to said test substrate.
- 10 2. The method of Claim 1, wherein said testing means further comprises one or more gel-initiating agents.
3. The method of Claim 2, wherein said gel-initiating agent comprises cationic salts.
- 15 4. The method of Claim 1, wherein said testing means further comprises one or more gelling agents.
5. The method of Claim 4, wherein said gelling agent is selected from the group
20 consisting of agar, Gelrite™, carrageenan, and alginic acid.
6. The method of Claim 1, wherein said microorganisms are in an aqueous suspension.
- 25 7. The method of Claim 6, wherein said aqueous suspension further comprises one or more gelling agents.
8. The method of Claim 4, wherein said gelling agent is selected from the group
30 consisting of agar, Gelrite™, carrageenan, and alginic acid.
9. The method of Claim 1, wherein said microorganisms are bacteria.

10. The method of Claim 9, wherein said bacteria are members of the Order *Actinomycetales*.

11. The method of Claim 1, wherein said microorganisms are fungi.

12. The method of Claim 1, wherein said testing substrates are selected from the group consisting of carbon sources and antimicrobials.

13. The method of Claim 1, wherein said testing means comprises a microplate.

14. A kit, comprising redox purple and one or more test substrates.

15. The kit of Claim 14, wherein said test substrates are selected from the group consisting of carbon sources and antimicrobials.

16. The kit of Claim 14 further comprising one or more gel-initiating agents.

17. The kit of Claim 16, wherein said gel initiating agent comprises cationic salts.

18. The kit of Claim 14, further comprising one or more gelling agents.

19. The kit of Claim 18, wherein said gelling agent is selected from the group consisting of agar, Gelrite™, carrageenan, and alginic acid.

20. The kit of Claim 14, further comprising a suspension of microorganisms.

21. The kit of Claim 14, further comprising a testing means.

22. The kit of Claim 22, wherein said testing means comprises a microplate.

23. A kit, comprising redox purple and one or more gelling agents.

24. The kit of Claim 23, wherein said gelling agent is selected from the group consisting of Gelrite™, carrageenan, and alginic acid.

25. The kit of Claim 23, further comprises one or more gel-initiating agents.

26. The kit of Claim 25, wherein said gel-initiating agent comprises cationic salts.

27. The kit of Claim 23, further comprising one or more test substrates.

28. The kit of Claim 27, wherein said test substrates are selected from the group consisting of carbon sources and antimicrobials.

29. The kit of Claim 23, further comprising a suspension of microorganisms.

30. The kit of Claim 23, further comprising a testing means.

31. The kit of Claim 30, wherein said testing means comprises a microplate.

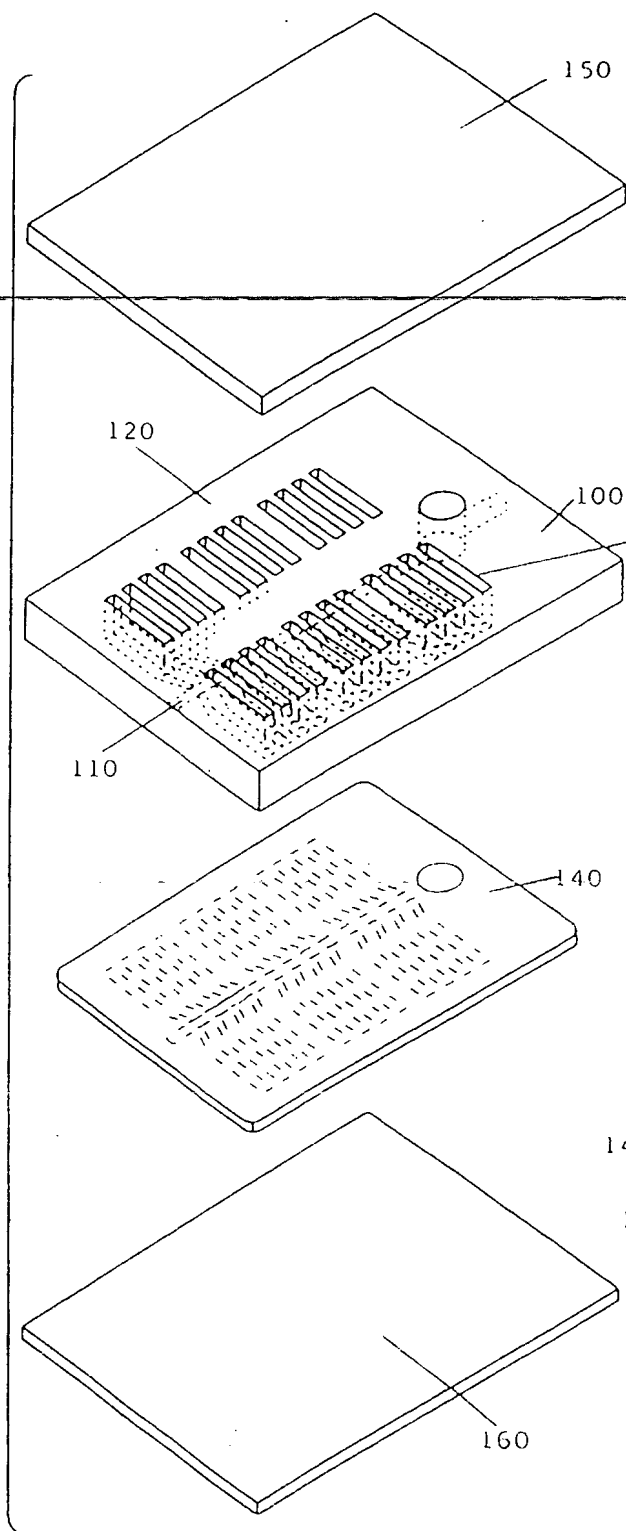


FIG. 1

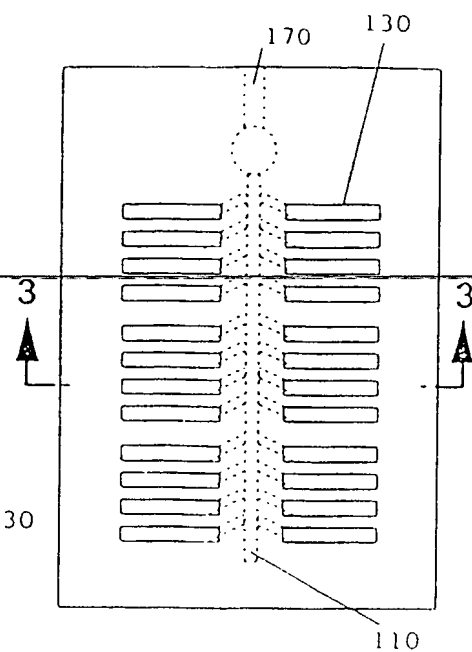


FIG. 2

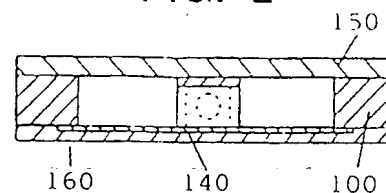


FIG. 3

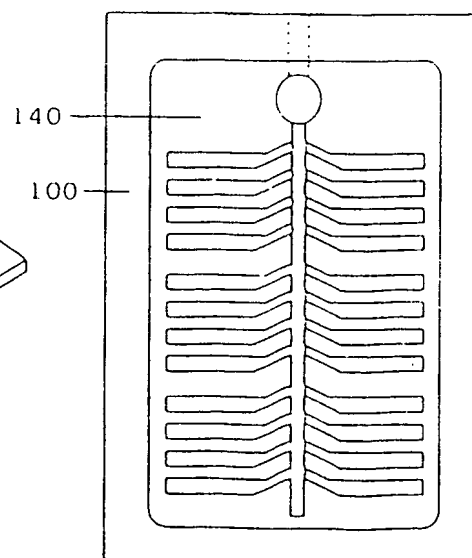
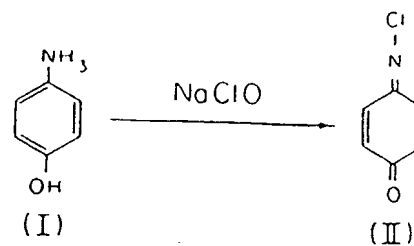


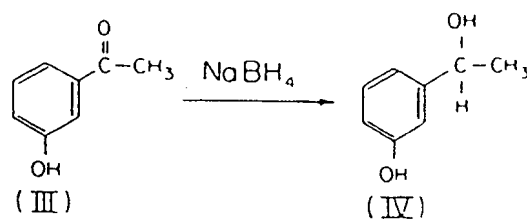
FIG. 4

FIGURE 5

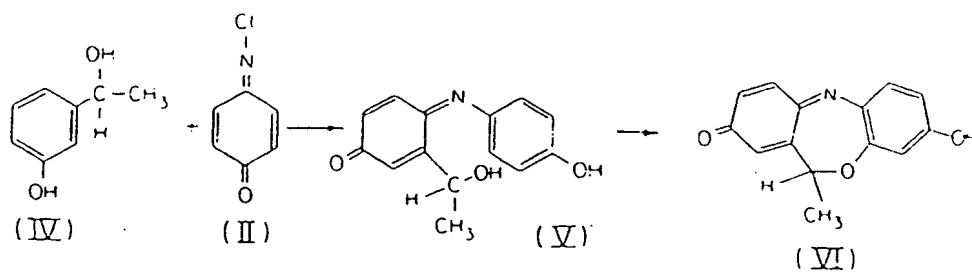
PANEL A



PANEL B



PANEL C





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/00, 1/02, 1/04, 1/18	A3	(11) International Publication Number: WO 98/26270 (43) International Publication Date: 18 June 1998 (18.06.98)
(21) International Application Number: PCT/US97/22601 (22) International Filing Date: 9 December 1997 (09.12.97) (30) Priority Data: 08/762,656 9 December 1996 (09.12.96) US	(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: BIOLOG, INC. [US/US]; 3938 Trust Way, Hayward, CA 94545 (US). (72) Inventors: BOCHNER, Barry, R.; 22 Coleport Landing, Alameda, CA 94502 (US). NALEWAY, John, J.; 3247 Lincoln Street, Eugene, OR 97405-1927 (US). (74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).	(88) Date of publication of the international search report: 3 September 1998 (03.09.98)	

(54) Title: GEL MATRIX WITH REDOX PURPLE**(57) Abstract**

The present invention is directed to methods and compositions for the characterization of various microorganisms. In particular, the present invention is suited for the characterization of commonly encountered microorganisms (*e.g.*, *E. coli*, *S. aureus*, etc.), as well as commercially and industrially important organisms from various and diverse environments. For example, the present invention is particularly suited for the growth and characterization of the actinomycetes and fungi.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/00, 1/02, 1/04, 1/18

US CL : 435/4, 29, 32, 34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 29, 32, 34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, JPO, EPO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3,963,442 A (<i>BULLARD</i> et al) 15 June 1976, col. 4, lines 28-46, col. 8, lines 47-49, and Table II.	1-31
Y, P	US 5,627,045 A (<i>BOCHNER</i> et al) 06 May 1997, abstract, col. 1, lines 1-16, col. 11, lines 44-48, and claims.	1-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 JUNE 1998

Date of mailing of the international search report

16 JUL 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized Officer

CHRISTOPHER TATE

Telephone No. (703) 308-0196

